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14. ABSTRACT
Hyper-activation of the PI3K-AKT-mTOR signaling pathway is frequently observed in advanced prostate cancer and has been suggested to promote survival of prostate cancer cells under androgen deprivation. The transcription factor C/EBP beta is expressed as three different translational isoforms from a single transcript and has been suggested to regulate mTOR activity. The longer LAP isoforms promote cell survival, growth arrest and differentiation whereas the LIP isoform can promote cell proliferation. The purpose of these studies were to evaluate the role of C/EBP beta translational isoforms in prostate cancer resistance to chemotherapy. The 4E/G interaction inhibitor (4E/Gi), which blocks cap-dependent translation, significantly upregulated the LAP isoforms in both PC3 and LNCaP cells. Suppression of C/EBP beta in PC3 cells increased mTOR activity and induction of LAP expression by 4E/Gi and bicalutamide treatment additively suppressed mTOR. We also evaluated the proteasome inhibitor bortezomib as another means to alter C/EBP isoforms, because it can also inhibit cap-dependent translation. Bortezomib increased the LAP:LIP ratio in LNCaP and PC3 cells and suppression of CEBPB sensitized these cells to bortezomib in vitro. PC3 xenografts deficient in CEBPB showed suppressed growth and were also sensitized to bortezomib administration. Our data suggest that CEBPB LAP isoforms suppress AKT-mTOR activity, but may protect prostate cancer cells from bortezomib. Conversely, increasing CEBPB LAP isoform levels by blocking cap-dependent translation may be an effective strategy to prevent transition to castrate-resistant prostate cancer or sensitize prostate cancer cells to mTOR inhibitors by suppressing AKT activity.

15. SUBJECT TERMS
mTOR, PI3K, C/EBP beta, androgen receptor, cap-dependent translation

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1. Introduction:

Central to the survival of prostate cancer (PCa) are the androgen receptor (AR) and phosphatidylinositol-3 kinase (PI3K)-AKT signaling pathways. Indeed, it has been reported that 50-70% of human prostate cancers have mutations in PI3K signaling, often through loss of phosphatase and tensin homolog (PTEN). AR gene amplification is also frequently observed in hormone refractory prostate cancer (HRPC). Combined blockade of PI3K and AR signaling is a strong apoptotic stimulus for PCa cells and shows promise for future therapy [1, 2]. Intriguingly, AR and PI3K-AKT signaling are reciprocal inhibitors of one another, but can both promote cell survival and proliferation in PCa. Inhibition of a single pathway leads to alternative survival by the other. PI3K-AKT signaling activates mammalian target of rapamycin (mTOR) activity, a critical regulator of cell growth and global protein synthesis. mTOR is often hyper-activated in human cancer due to high metabolic demand of rapidly growing tumors. mTOR is a kinase that forms two distinct complexes in the cell referred to as mTOR complex (mTORC) 1 and -2. mTORC1 is a complex between mTOR, raptor and LST8, which functions to negatively regulate autophagy and promote protein synthesis [3]. mTORC2 is comprised of mTOR, rictor, LST8 and SIN1, whose best characterized functions are activating AKT and actin cytoskeletal organization [4]. Although a promising therapeutic target, rapamycin and rapalogs have only achieved modest clinical benefit, indicating PCa cells initiate compensatory mechanisms for survival even during decreased AR and mTOR signaling [5, 6]. Members of the CCAAT/enhancer binding protein (C/EBP) family are regulated through translation mechanisms by mTOR activity and have been linked to PCa survival and metastatic gene expression [7, 8]. C/EBPα and C/EBPβ (C/EBPα/β) are both transcription factors that function to exert cell cycle control and terminal differentiation. More recently, these factors have been recognized to suppress both the intrinsic and extrinsic apoptotic pathways through the expression of MCL-1, cFLIP, BCL-2 and BCL-xL [7, 9]. Both members are expressed as a single intronless transcript and truncated translational isoforms are generated by leaky ribosomal scanning. Activity of mTORC1, eukaryotic initiating factor 4E (eIF4E) or eIF2α causes the ribosome to skip the first AUG and initiate translation of the truncated isoforms [8, 10, 11]. Importantly, it has been shown that truncated and long C/EBPα/β isoforms have differential cellular function. Truncated C/EBPs lack transcriptional activation domains and suppress gene transcription by heterodimerizing with full-length isoforms or directly binding to target gene promoters and recruiting HDACs. the long isoforms activate transcription in most contexts and are more functionally linked with survival, cell cycle arrest and terminal differentiation. [10, 12, 13]. In contrast, truncated isoforms function to increase cell growth and act as silencers of tumor suppressor pathways. For example, mutation of the CEBPα gene locus, so that only the truncated p30 isoform is expressed, results in development of acute myeloid leukemia [15]. C/EBPβ LIP expression increases mammary epithelial cell proliferation and antagonizes anti-proliferative signals from TGF-β in breast cancer cells [16]. Because truncated C/EBPs antagonize the activity of full length isoforms, blocking LIP or p30 translation with mTOR or cap-dependent translation inhibitors could promote full length C/EBP translation as a mechanism for PCa cells to suppress proliferation, but avoid apoptosis, e.g. by increasing anti-apoptotic gene expression. The purpose of this research project is to determine the functional roles of C/EBPα/β translational isoforms in PCa progression to CRPC, tumor growth and survival and resistance to androgen deprivation.

2. Keywords:

mTOR, PI3K, C/EBP beta, androgen receptor, cap-dependent translation

3. OVERALL PROJECT SUMMARY:

Current Objectives

The overall objectives of this fellowship award is to train and develop the career of Dr. Barakat so that he can function as an independent prostate cancer investigator and investigate the mechanisms of C/EBP translational isoforms in the survival of androgen sensitive and castrate-resistant prostate cancer cells.

The research specific objectives of the award are as follows:

- Evaluate C/EBPα/β translational isoform expression in PCa cell lines treated with pharmacological inhibitors of the PI3K-AKT-mTOR signaling pathway. Months 1-8. 100% completed.

- Evaluate proliferation and survival in PCa cell lines with combined C/EBPα/β KD or over-expressing individual C/EBP translational isoforms. Months 9-13. 100% completed.

- Studies with engineered prostate cancer cell lines evaluating tumor growth rates in NOG mice and sensitivity to chemical inhibitors. Months 13-24. 100% completed.

Training-specific goals for this award period were as follows:

- Attend selected prostate cancer specific research seminars. 100% completed

- Attend 2015 American Association for Cancer Research annual meeting. Completed April 2015.

- Present ongoing research at Prostate Cancer Research Day. Completed February 2015.

- Attended the 2015 Progenitor Cell Biology Consortium Workshop: CRISPR/Cas9 Genome Engineering for Human Progenitor Cell Biology and Translation. UT Southwestern Medical Center, Dallas, TX.

- Publication of results in peer-reviewed scientific journals. Completed

- Submitted revised K99/R00 Pathway to Independence award. Completed. Ultimately, this award was not funded. At the next opportunity, I plan to submit a K22 transition award to the NCI.

Results

C/EBPα and -β are transcription factors that are expressed from an intronless transcript. Truncated isoforms, C/EBPα p30 and C/EBPβ LIP, which lack transcriptional activation domains are generated by translation from in-frame, down-stream start codons and can exert dominant-negative effects by heterodimerizing with full-length isoforms [8]. Because previous studies had indicated that cap-dependent translation and mTOR were critical drivers of C/EBPα/β truncated isoforms, we therefore evaluated the effect of pharmacological inhibitors of PI3K, mTOR or cap-dependent translation on C/EBP translational isoforms by Western blot analysis. We treated the PTEN-null LNCaP and PC3 cell lines for 6 hours with PI3K inhibitor Ly294002 (25 μM), mTORC1 inhibitor RAD001 (100nM), mTORC1/2 inhibitor INK128 and 4E/Gi (25 μM), which blocks cap-dependent translation by disrupting

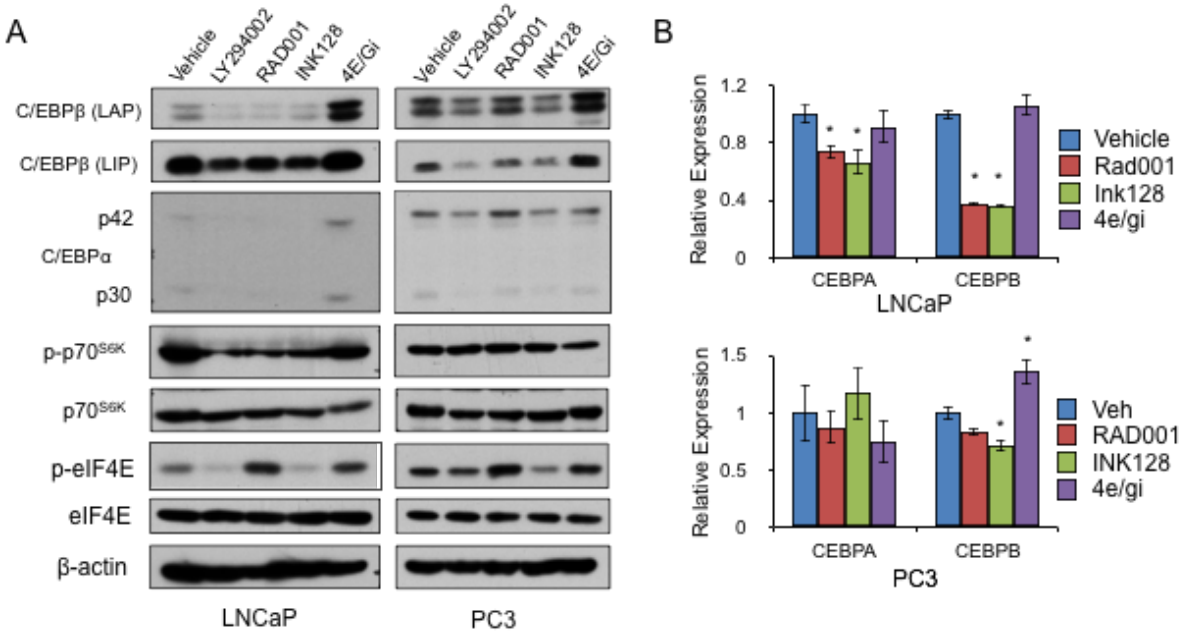

Figure 1: Cap-dependent translation suppresses C/EBP full-length isoform expression in PTEN-null prostate cancer cell lines. Western blot **(A)** and qPCR **(B)** analysis of PC3 and LNCaP cells treated for 6 hours with 25μM LY294002, 100nM RAD001, 100nM INK128 or 50μM 4E/Gi. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

the interaction between eukaryotic initiation factor 4E (eIF4E) and eIF4G. In LNCaP and PC3 cells, blockade of mTOR or PI3K lead to down regulation of all C/EBP isoforms. Intriguingly, treatment with 4E/Gi lead to induction of C/EBPβ LAP isoforms in LNCaP cells with preferential induction of LAP. Further, 4E/Gi increased expression of all three isoforms in PC3 cells (Fig. 1a). Expression of C/EBPα was almost undetectable in LNCaP cells and modest in PC3 cells, indicating that it may not play a strong role in response to mTOR inhibition in prostate cancer cells. Blocking PI3K or mTOR decreased phosphorylation of p70S6K in LNCaP cells, but had no effect in PC3 cells. Intriguingly, TORC1/2 blockade with INK128 blocked phosphorylation of eIF4E in both lines, indicating that INK128 has a strong effect on the inhibition of cap-dependent translation. We next evaluated C/EBPα/β gene expression under similar conditions in these cell lines by quantitative real-time PCR (qPCR) to determine whether the changes in C/EBP expression were attributable to altered mRNA levels. Blockade of mTOR by RAD001 or INK128 lead to significant down-regulation of C/EBPα/β gene expression and 4E/Gi had no effect on expression of these genes in LNCaP cells (Fig 1B). Ink128 down-regulated C/EBPβ levels and 4E/Gi modestly up-regulated CEBPB transcript levels in PC3 cells (Fig. 1B). mTOR inhibitors did not have a selective effect on C/EBPβ translational isoform expression and in agreement with these results, we observed an overall decrease in C/EBPβ mRNA and protein levels in LNCaP and PC3 cells. These results indicate that cap-dependent translation rather than mTOR activity plays a crucial role in regulating the LIP:LAP isoform ratio in prostate cancer cell lines. Because we found that

C/EBPα expression was minimally expressed in these cell lines, we focused our efforts on C/EBPβ for the remaining studies.

Bortezomib promotes C/EBPβ LAP isoforms and suppresses cap-dependent translation.

Because the 4E/Gi did not induce cell death as a single agent in PC3 cells and 4E/Gi is not usable as an *in vivo* drug due to low bioavailability (not shown), we decided to evaluate the effects of other chemotherapy drugs which could potentially suppress cap-dependent translation and alter the C/EBPβ translational isoform ratio. The proteasome inhibitor bortezomib inhibits cap-dependent translation by dephosphorylation of eIF4E binding protein 1 and activates a cellular stress response that promotes autophagy [22, 42]. Bortezomib has been tested in prostate cancer patients, but the clinical data did not show a significant improvement in patient benefit when administered in combination with hormonal therapy. However, a recent study evaluating microarray data from younger patients (<65 years of age) with localized prostate cancer and treated by radical prostatectomy revealed that gene sets representing proteasome subunits and protein catabolism are the strongest predictors of metastatic progression [35]. Further, it has been shown that bortezomib can up-regulate C/EBPβ gene and protein expression [27]. We therefore tested whether bortezomib could alter the C/EBPβ LAP:LIP isoform ratio in PCa cell lines. 24 hours after treatment, we observed up regulation of C/EBPβ transcript and proteins levels in both LNCaP and PC3 cells (Fig 2A and B). Further, there was a dramatic increase in the LAP isoform levels in both of these lines (Fig 2A). When we analyzed PC3 cells that had been treated with 50nM bortezomib by time course analysis, we observed a decrease in the inactive hyperphosphorylated 4EBP1, as well as the AKT/mTOR inhibitor REDD1 (Fig. 2C). These data demonstrate that bortezomib can increase the C/EBPβ LAP:LIP translational isoform ratio in prostate cancer cell lines similar to 4E/Gi and suppress cap-dependent translation.

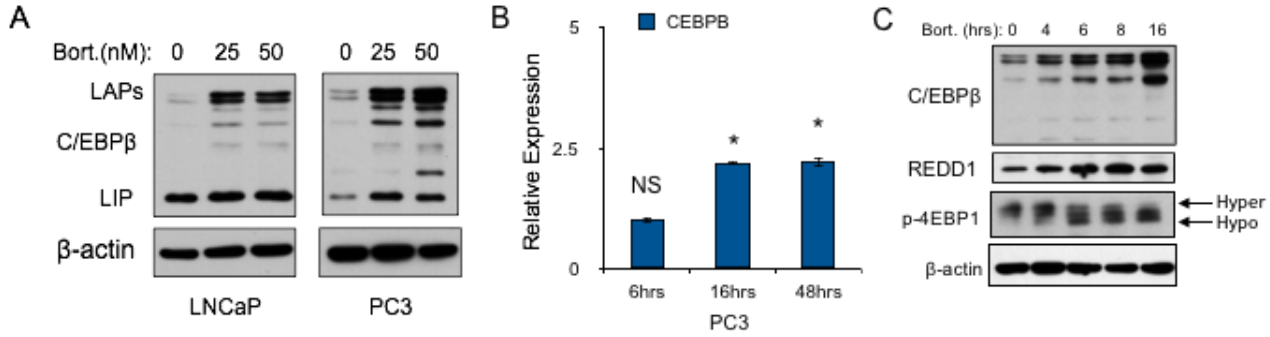

Figure 2. Bortezomib increases C/EBPβ LAP isoforms in PCa cell lines. **(A)** Western blot analysis of C/EBPβ in LNCaP and PC3 cells treated with escalating doses of bortezomib for 24 hours. **(B)** Analysis of *REDD1* and *CEBPB* gene expression in PC3 cells by qPCR. **(C)** Time course analysis of 4EBP1 phosphorylation, REDD1 and C/EBPβ in PC3 cells treated with 50nM bortezomib by Western blot analysis. * $p < 0.05$. Error bars represent average of three experiments

C/EBPβ LAP:LIP isoform ratio regulates autophagy in prostate cancer cells

Because C/EBPβ is a transcriptional regulator of autophagy and bortezomib induces the activation of autophagy, we hypothesized that up-regulation of C/EBPβ LAP isoforms promote PCa autophagy in response to bortezomib. We first tested whether over expression of C/EBPβ could activate autophagy. We first ectopically expressed murine C/EBPβ, which shares 98.8% similarity to the human C/EBPβ bZIP domain and 80-100% similarity to the human trans-activation and regulatory domains, in the PC3 PCa cell lines using the PB-TRE Tet-on PiggyBac inducible expression vector we had used previously to obtain LNCaP cells harboring doxycycline-inducible C/EBPβ [52]. Induction of PiggyBac-CEBPB (PB-CEBPB) in PC3 cells led to a decline in the total levels of LC3-I and LC3-II and p62 compared to cells expressing the empty PB-TRE vector (Fig. 3A, left). Decreases in p62 levels typically indicate increased autophagic degradation, while changes in LC3-II can imply blockade or activation of autophagosome formation [53]. We further evaluated other proteins involved in autophagosome nucleation and elongation and found increases in ATG3, ATG5, ATG7 and Beclin-1, suggesting that autophagy is increased upon induction of C/EBPβ (Fig. 3A, right). In contrast with these results in PC3

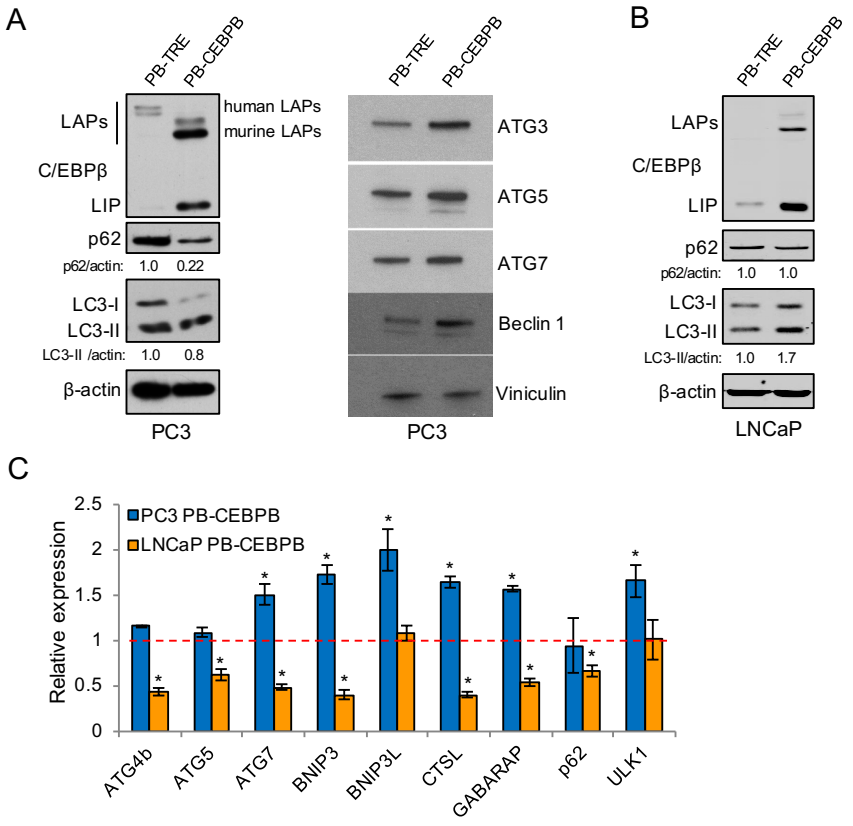

Figure 3. C/EBPβ isoforms LAP and LIP differentially regulate autophagy in PCa cells. **(A)** PC3 cells stably transduced with PB-TRE or PB-CEBPB were treated for 72 hrs with 0.5 μg/ml doxycycline and cell lysates were analyzed by Western blotting for the indicated proteins. Numbers below the blots indicate the relative band density normalized to β-actin. **(B)** LNCaP cells stably transduced with PB-TRE or PB-CEBPB were analyzed similarly for C/EBPβ, p62, LC3, and β-actin. **(C)** PC3 or LNCaP cells transduced with PB-TRE or PB-CEBPB were cultured similarly followed by qPCR analysis for the indicated genes. The dashed line represents gene expression levels in control cell lines expressing PB-TRE. Bar graphs represent the average of three experiments, error bars represent standard error of the mean (SEM). (*p<0.05).

cells, LNCaP cells showed increased total levels of LC3-I and LC3-II and no change in p62 in response to C/EBPβ induction (Fig. 3B). Of note, ectopic C/EBPβ is predominantly expressed as the shorter, dominant inhibitory LIP isoform in LNCaP cells as is endogenous C/EBPβ. Thus induction of CEBPB in LNCaP cells may reduce C/EBPβ trans-activation activity to impair autophagy. As C/EBPβ was shown to directly regulate autophagic genes at the transcriptional level [54, 55], we also evaluated the expression of several such genes by quantitative real-time PCR (qPCR). We found a modest increase in *CTSL*, *GABARAP*, *ATG7*, *BNIP3*, *BNIP3L* and *ULK1* mRNAs in PC3 cells and that nearly all of these genes were suppressed upon ectopic C/EBPβ expression in LNCaP cells (Fig. 3C). These data suggest that the C/EBPβ LIP isoform suppresses autophagy and the C/EBPβ LAP isoform activates autophagy.

C/EBPβ LAP promotes autophagy by augmenting REDD1 expression

Although C/EBPβ is reported to broadly regulate the expression of autophagy genes in the liver [30], we did not observe differential expression of all genes analyzed in PC3 cells over-expressing C/EBPβ. Recently, it was shown that REDD1 could promote autophagy by suppressing ATG4b-mediated LC3-II delipidation to LC3-I, an effect most critical during periods of metabolic stress [36]. This function of REDD1 prevents recycling of LC3-II and leads to greater turnover of autophagosomes. REDD1 can also inhibit mTORC1, which suppresses autophagy by phosphorylating ULK1 and ATG13, upstream autophagy regulators [23]. C/EBPβ was previously shown to cooperate with ATF4 to promote *REDD1* transcription by binding to a non-consensus site centered 1,004 bp upstream from the *REDD1* transcription start site (TSS), but only in cells under oxidative stress [39,40]. We evaluated *REDD1* gene expression changes in PCa cells expressing shRNA targeting *CEBPB* by qPCR. In contrast to cells over-expressing C/EBPβ, we found that none of the genes that we had analyzed in Figure 1 were affected by C/EBPβ KD in LNCaP or PC3 cells (Fig. 4A). However, in PC3 cells with *CEBPB* deficiency, we found significant 2-fold down-regulation of *REDD1* transcript levels (Fig 4A). We also found that LNCaP cells showed increased *REDD1* upon shCEBPB induction, likely due to decreased expression of the dominant-inhibitory LIP isoform (Fig. 4A). Notably, *REDD1* mRNA showed stronger up-regulation upon C/EBPβ over-expression in PC3 cells than other autophagy genes analyzed (Fig. 4B). Conversely, over-expression of C/EBPβ in LNCaP cells suppressed *REDD1* transcript levels (Fig. 4B).

As we observed that *REDD1* expression correlated with full-length C/EBPβ levels in the absence of oxidative stress, we analyzed the human *REDD1* promoter to identify additional binding sites for C/EBPβ. We found a site matching the C/EBPβ consensus at -603 bp and a near-consensus site at -99 bp. To test whether C/EBPβ binds to these regions, we subjected nuclear extracts from PC3 cells to

chromatin immunoprecipitation (ChIP). Using two different primer pairs, each flanking one of the putative C/EBPβ binding sites, we observed between 6 and 8-fold greater levels of amplified product in extracts precipitated with anti-C/EBPβ antiserum relative to normal rabbit IgG (Fig. 4C), indicating that endogenous C/EBPβ binds to these regions of the *REDD1* promoter.

C/EBPβ regulation of REDD1 suggests that C/EBPβ is not only critical for autophagosome-lysosome fusion, but also might play a role in autophagosome maturation. Residual REDD1 present upon shCEBPB-mediated *CEBPB* KD must be sufficient to prevent a complete block at this step in autophagy. Knockdown of REDD1 was problematic, as all siRNAs tested also reduced C/EBPβ (not shown), and of note REDD1(-/-) MEFs show greatly reduced autophagosome formation [36]. Therefore, to further test whether C/EBPβ drives early autophagy via *REDD1*, we transiently over-expressed REDD1 in PC3 cells. We found that expression of REDD1 from the pCMS-EGFP-REDD1 vector [39] increased LC3-II in PC3 cells harboring shNTV, as predicted from its role in early autophagy, but mildly reduced LC3-II in shCEBPB cells, compared to controls expressing the pMax-GFP plasmid (Fig. 4D). These results suggest that C/EBPβ promotes autophagy in part by regulating *REDD1* expression.

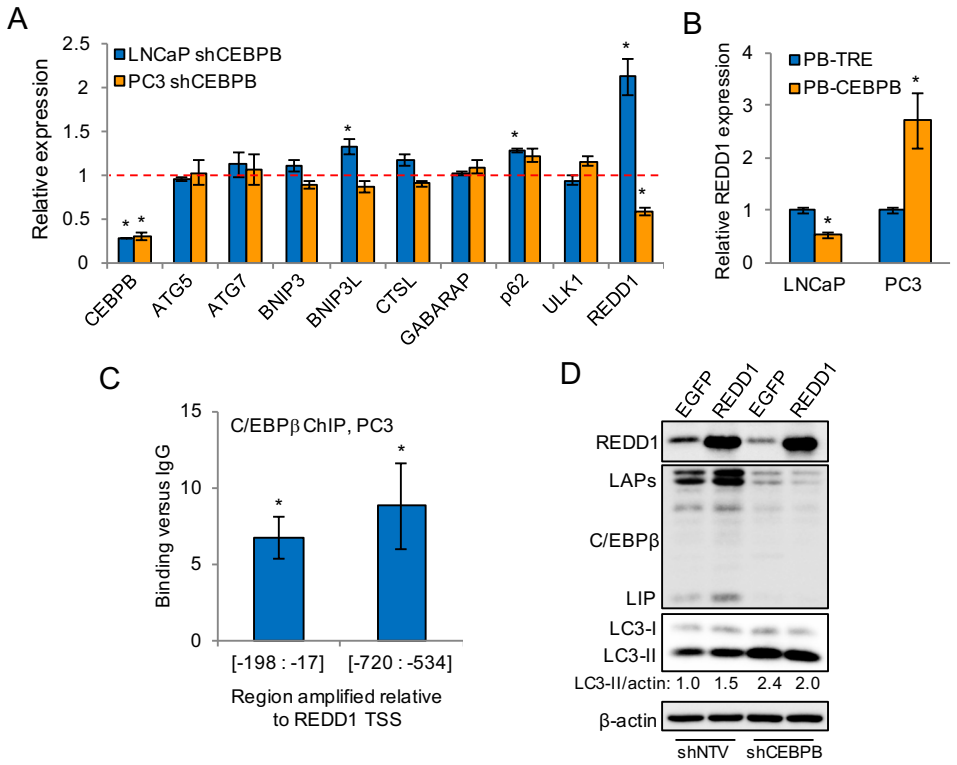

Figure 4. C/EBPβ promotes autophagy in PC3 cells by regulation of *REDD1* expression. **(A)** qPCR analysis of autophagic genes in PC3 and LNCaP cells expressing shRNA targeting *CEBPB*. The dashed line represents gene expression levels in control cell lines expressing shNTV. **(B)** *REDD1* gene expression in cells expressing C/EBPβ from the PB-CEBPB vector or harboring the control PB-TRE vector after 72 hr culture with doxycycline. **(C)** qPCR analysis, using two primer pairs, of genomic DNA extracted from PC3 cell chromatin following immunoprecipitation with anti-C/EBPβ antibody or IgG control. Fold-increase in C/EBPβ binding versus IgG is shown. (D) PC3 cells expressing shNTV or shCEBPB were transiently transfected with pMax-GFP or pCMS-EGFP-REDD1, and 48 hours later whole cell lysates were subject to Western blot analysis for the indicated proteins. Data presented is representative of three independent experiments. Bar graphs represent the average of three experiments, error bars represent SEM. (*p<0.05).

C/EBPβ promotes autophagy following bortezomib treatment

Previous reports suggest that proteasome inhibitors increase expression and activity of C/EBPβ [27]. As proteasome inhibitors also activate autophagy [42, 56], we tested whether C/EBPβ is required for autophagy following bortezomib treatment. Gene expression analysis of several autophagy genes revealed that *ULK1* and *p62* were markedly increased 16 hours after bortezomib treatment of PC3 cells (Fig. 5A). *ATG5* and *ATG4B* were unchanged and *ATG7* and *REDD1* were mildly upregulated by bortezomib. The changes in *p62* and *ULK1* were not mediated by C/EBPβ because shCEBPB cells showed the same increase in gene expression upon bortezomib treatment (Fig. 5B). Treatment of LNCaP or PC3 cells with bortezomib for 24 hours markedly increased C/EBPβ (LAP), LC3-II, and p62 protein expression (Fig. 5C). To further evaluate the effects of bortezomib on PCa cell autophagy, we analyzed LC3 and p62 by time course analysis. We found that REDD1 increased from 4-8 hours following 25 nM bortezomib treatment, but then decreased between 8 and 16 hours (Fig. 5D). LC3-II and p62 protein

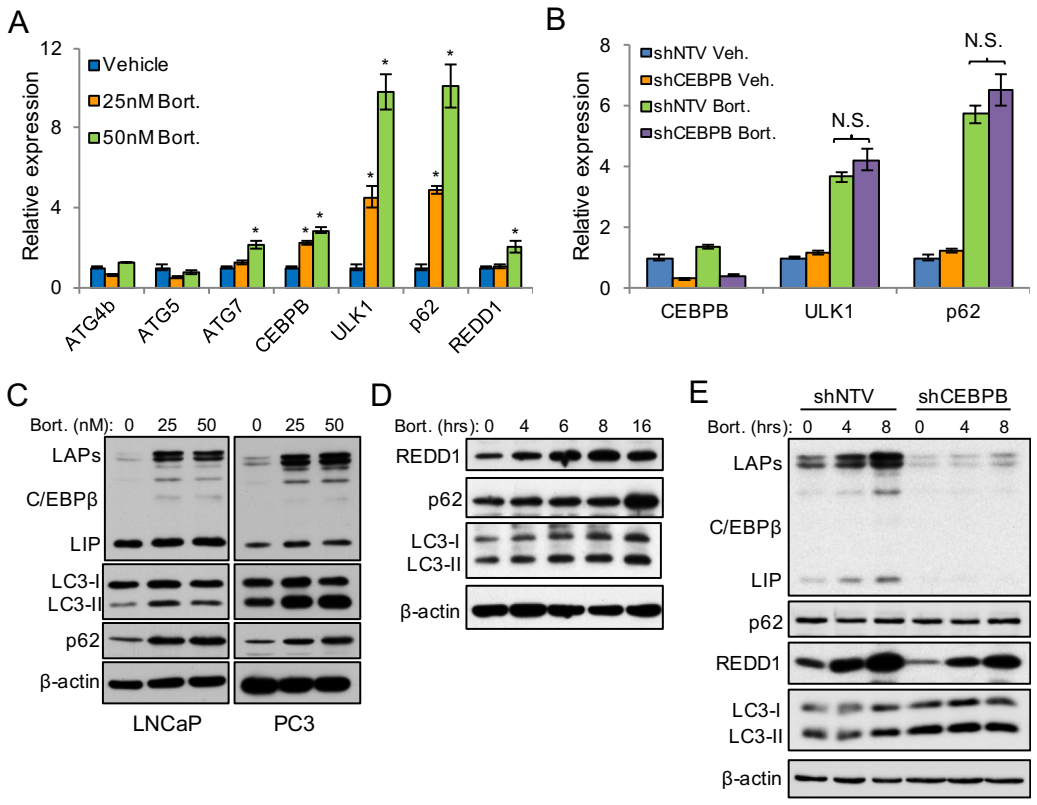

Figure 5. C/EBPβ promotes autophagy driven by bortezomib challenge. **(A)** qPCR analysis for indicated mRNAs from PC3 cells that had been treated with bortezomib or vehicle for 16 hrs. **(B)** qPCR analysis of autophagy genes in PC3 cells expressing shNTV or shCEBPB and that had been treated for 16 hrs with 25 nM bortezomib or vehicle. **(C)** LNCaP or PC3 parental cells were treated for 24 hrs with the indicated concentrations of bortezomib and cell lysates were then subjected to Western blot analysis. **(D)** PC3 cells were treated with 25 nM bortezomib for the indicated times and cell lysates were then analyzed by Western blot analysis for indicated proteins. **(E)** PC3 cells expressing NTV or CEBPB shRNAs were treated with 50 nM bortezomib for the indicated times and cell lysates were again analyzed by Western blot analysis. Numbers below blots indicate relative density after normalizing to β-actin. Bar graphs represent the average of three experiments, error bars represent SEM. (*p<0.05).

levels showed the strongest increase 16 hours after treatment. Lastly, we tested whether C/EBPβ was critical for autophagy following bortezomib exposure. We treated PC3 cells expressing shCEBPB with bortezomib and evaluated autophagy markers by time course analysis. C/EBPβ deficiency decreased REDD1 expression and prevented increases in LC3-II, though shCEBPB cells had higher basal levels of LC3-II (Fig. 5E). These data indicate that bortezomib promotes autophagy and that C/EBPβ is critical for activating autophagy early after bortezomib treatment.

C/EBPβ LAP promotes prostate cancer survival by augmenting autophagy.

We next evaluated the role of C/EBPβ in regulating prostate cancer survival in response to bortezomib. shNTV and shCEBPB LNCaP cells were seeded in 96-well plates and treated with escalating doses of bortezomib for 48 hours. We evaluated cell viability by WST-1 assay and observed a significant decrease in the IC50 value of cells deficient in C/EBPβ indicating increased sensitivity to bortezomib (Fig. 6A). We also evaluated PC3 cells under similar conditions, but did not observe a significant difference in IC50 value (Fig. 6B). However, this assay may not have been suitable for determining drug sensitivity in these cells because values from treated cells are normalized to vehicle treated controls and we observed a substantial decrease in PC3 growth upon suppression of C/EBPβ (fig 7a and b). This would suggest that the response to drug treatment is actually greater than normalized values in cells lacking C/EBPβ. We therefore evaluated cell death by trypan blue exclusion in cells treated with 25 and 50nM bortezomib for 24 hours. We observed a significant increase in cell death in shCEBPB cells treated with 25nM (17.2% vs 34.5%) and 50nM (22.8% vs 45.8%) bortezomib (Fig 6C). To evaluate whether autophagy contributed to increased cell death in shCEBPB cells we treated cells with chloroquine (CQ) in combination with bortezomib. Chloroquine is a weak base that accumulates in the acidic compartments of late endosomes and lysosomes, alkalinizes these structures, and subsequently inhibits autophagosome-lysosome fusion [43, 44]. We found that there was no difference in shNTV versus shCEBPB cell death in cultures treated with the combination of CQ and bortezomib, indicating that increased death in shCEBPB cultures was due to a block in autophagy (Fig 6D). These results demonstrate that C/EBPβ promotes resistance to bortezomib in prostate cancer cells.

C/EBPβ promotes prostate cancer cell growth and sensitivity to bortezomib in vivo.

Next, we generated a pair of TALEN expression vectors with targeting sequences to the human CEBPB gene locus and a donor plasmid containing CEBPB homology arms flanking a neomycin resistance cassette (See Barakat et al., 2016 supplementary figure 1). The region between the homology

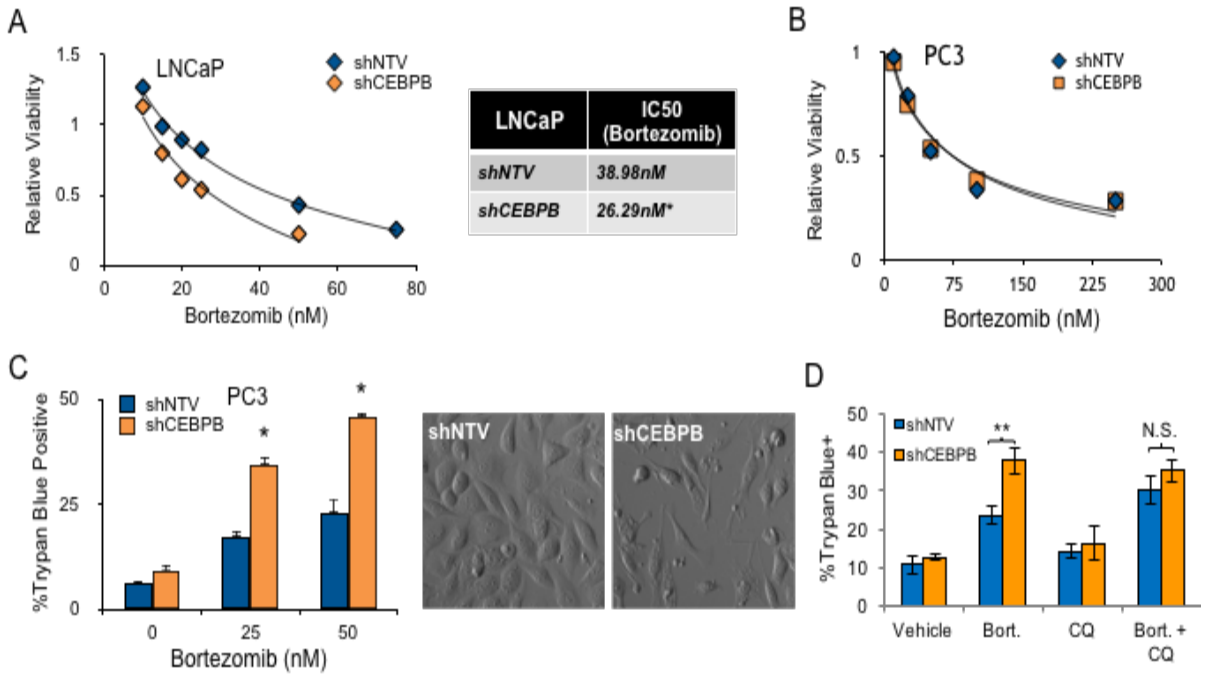

Figure 6. Suppression of C/EBPβ promotes cell death in response to bortezomib in prostate cancer cell lines. (A) Analysis of PCa cell sensitivity to bortezomib by WST-1 assay in LNCaP (A) and PC3 cells (B) treated with escalating doses of bortezomib for 48 hours. **(B)** Quantification of PC3 death by trypan blue exclusion in cultures treated for 24 hours with 0, 25 and 50nM bortezomib. **(C)** Western blot analysis of BCL2 expression in PC3 cultures treated with 25nM bortezomib for 0, 6 and 16hrs. **(D)** Quantification of PC3 death by trepan blue exclusion in combination with chloroquine. Bar graphs represent the average of three experiments. * $p<0.05$; ** $p<0.025$

arms within the CEBPB gene contains the TALEN targeting sites. We transfected PC3 cells with these vectors and 48hrs later added neomycin (250µg/ml) to the medium. Cells were incubated for 4 days in neomycin before being split into 96-well plates for neomycin resistant sub-clones. Genomic DNA from several sub-clones were analyzed by PCR with primers designed to amplify the deleted region of CEBPB. We identified one sub-clone which was negative for amplification of this region. However, we were still able to detect C/EBPβ protein in lysates collected from this sub-line (Fig 7a). A control sub-line, which was not treated with neomycin and still showed amplification of the CEBPB region by PCR was utilized as control (TALEN control). Subsequent attempts to generate cell lines with complete knockout were unsuccessful. However, these cells with partial loss of C/EBPβ may still be useful for the study of CEBPB in prostate cancer.

We next characterized the growth rates of TALEN and shCEBPB knockdown cells relative to their respective controls. We evaluated cell growth by manually counting cells with a hemacytometer 2 and 4 days after plating and found that suppression of C/EBPβ dramatically decreased the rate of PC3 cell growth by 2.5-fold in shCEBPB and >3-fold in TALEN KD cells (Fig. 7B). Consistent with this finding, we also evaluated clonogenic growth and observed a decrease in colony number in cultures deficient in C/EBPβ (Fig. 7C).

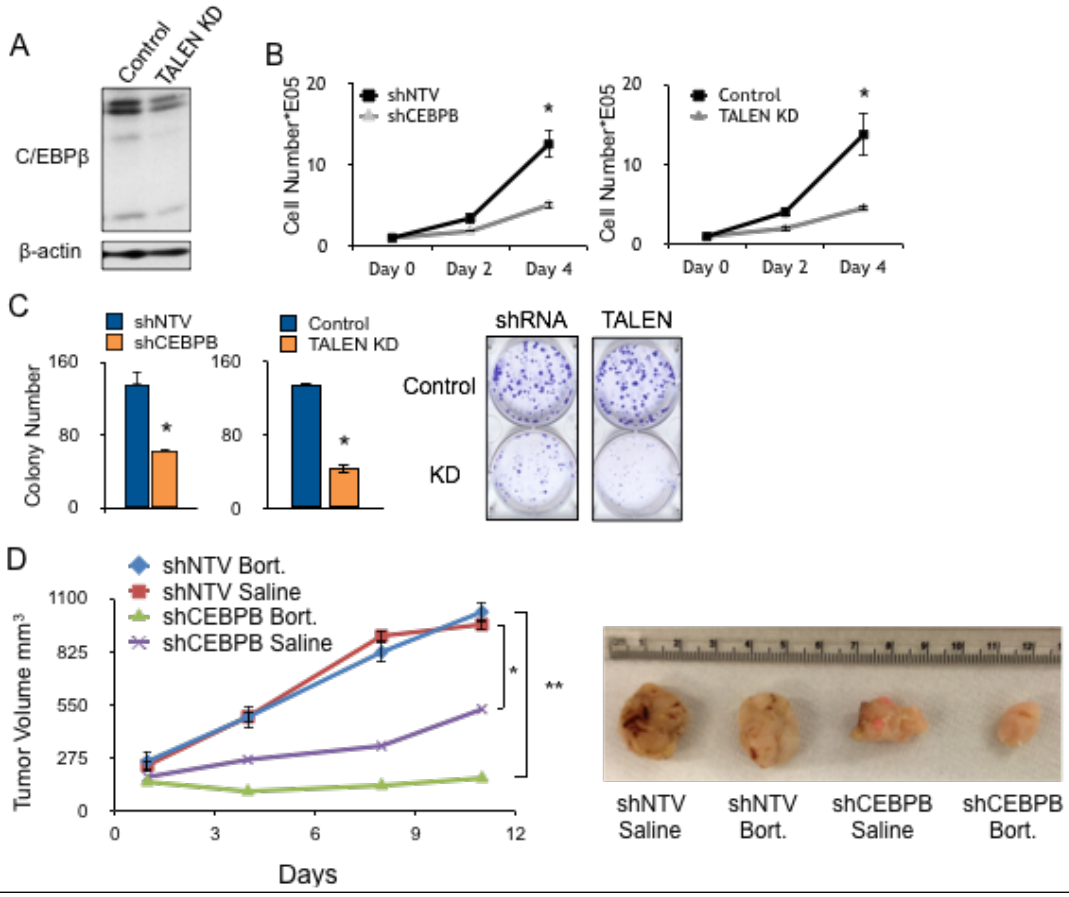

Figure 7. C/EBPβ deficiency suppresses PC3 growth and improves *in vivo* efficacy of bortezomib. (A) Growth of indicated PC3 lines after 3 and 5 days of culture (n=3). **(B)** Quantification of clonogenic growth of the indicated PC3 lines 12 days after seeding in 6-well plates at 200 cells/well. Crystal violet staining of representative wells (top) and mean colony numbers (bottom) are shown. **(C)** PC3 cells were incubated for 72 hrs in 0.5 µg/ml doxycycline prior to treatment with the indicated concentrations of bortezomib for 24 hrs. Quantification of cell viability by Trypan blue dye exclusion is shown (left). Representative phase contrast images showing shNTV and shCEBPB PC3 cells that had been treated with 50 nM bortezomib for 24 hrs (right). **(D)** Quantification of cell viability by Trypan blue dye exclusion in PC3 cultures that had been treated with the indicated drugs for 24 hrs. Bort - 25 nM bortezomib; CQ - 50 uM chloroquine. **(E)** Quantification of PC3 tumor growth in NSG mice treated with vehicle or bortezomib (1 mg/kg) on days 1, 4 and 8. (n = 7). Image to the right of the figure shows representative tumors from the indicated groups. Statistically significant differences were determined by linear regression analysis. Alpha adjusted by Holm-Bonferroni correction. Bar graphs represent the average of three experiments, error bars represent SEM. (*p<0.05; **p<0.025; ***p<0.0167).

Lastly, we evaluated the role of C/EBPβ in prostate tumor growth and sensitivity to bortezomib. We subcutaneously engrafted NSG mice with shNTV or shCEBPB PC3 cells in matrigel and when tumor reached sizes between 100 and 300mm^3 mice were placed on a doxycycline-laced animal feed for two days and then received IP injection of bortezomib (1mg/kg) or vehicle (DMSO 1: 5000 in saline) on days 1, 4 and 8. Tumor volume was evaluated by caliper measurement. We found that suppression of C/EBPβ significantly reduced the growth rate of prostate tumors (Fig. 7D). Significantly, we also found that administration of bortezomib did not significantly affect the growth rate of control shNTV tumors and that suppression of C/EBPβ sensitized these tumors to bortezomib treatment. These results demonstrate that C/EBPβ promotes the growth of castrate-resistant prostate cancer and resistance to bortezomib *in vivo*.

C/EBPβ regulates mTOR activity and CRPC progression.

Because we found that C/EBPβ LAP promotes REDD1, a negative regulator of mTOR and AKT activation, we next tested whether altered expression of C/EBPβ could affect mTOR activity. 72 hours after treating shCEBPB PC3 cells with 0.5μg/ml doxycycline, we found that phosphorylated p70S6K was increased relative to shNTV control cells. Conversely, over-expression of C/EBPβ decreased p-p70S6K levels, indicated that mTOR activation was suppressed (Fig. 8A). Because PC3 cells have predominant LAP expression, the effects of C/EBPβ on mTOR suppression could be attributable to LAP. We hypothesized that C/EBPβ LIP activates mTOR. We have previously found that the androgen sensitive cell lines LAPC4 and LNCaP exhibited predominant LIP expression and this isoform ratio was maintained after induction of C/EBPβ by anti-androgens bicalutamide and flutamide (Fig 8B). It has been reported that the anti-androgen enzalutamide can increase the activity of AKT and possibly mTOR [1]. We therefore tested whether C/EBPβ knockdown could repress mTOR activity following enzalutamide treatment in LNCaP PCa cells. Enzalutamide treatment increased 4EBP1 phosphorylation and did not affect p70S6K phosphorylation 72 hours after treatment in shNTV and shCEBPB cells (Fig. 8C). However, total levels of 4EBP1 increased in shNTV and shCEBPB cells. Unexpectedly, we found that C/EBPβ knockdown had no effect on p70S6K and decreased total levels of 4EBP1. These results suggest that LIP represses cap-dependent translation without affecting mTOR activity.

To determine whether C/EBPβ levels correlate with human prostate cancer progression we interrogated the Oncomine database. In the Grasso et al data set (26) that included gene expression patterns from 28 benign prostate tissues, 59 localized PC, and 35 CRPC samples CEBPB expression was significantly ($p=1.9x10-6$) elevated in CRPC compared with localized disease (Fig. 8D). Because C/EBPβ up regulation was associated with progression to CRPC, we next tested whether C/EBPβ played a role in the development of castrate-resistant growth of PCa tumors in a mouse xenograft model. shNTV or shCEBPB LNCaP cells were subcutaneously engrafted into male NSG mice and when tumors reached a volume between 100 and 300mm^3 animals were put on a doxycycline-laced animal feed and surgically castrated seven days later. Tumor volume was monitored weekly by caliper measurement for 8-weeks (Fig. 8E). We observed significant suppression of CRPC growth in xenografts expressing shC/EBPβ ($p<0.001$). These results suggest that C/EBPβ LIP is a critical determinant of CRPC progression, but does not promote mTOR activity in androgen sensitive cells.

Discussion:

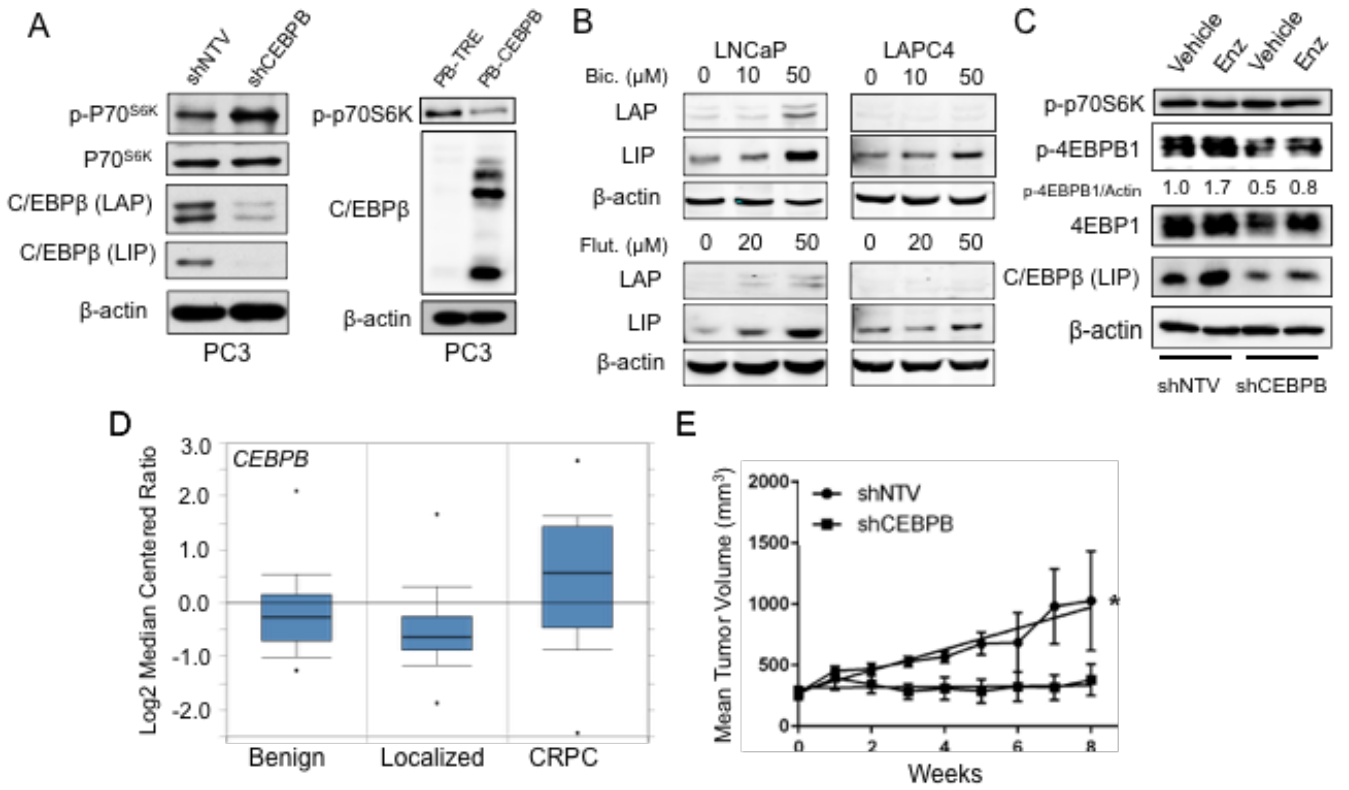

Figure 8. C/EBPβ regulates mTOR activation, 4EBP1 levels and promotes CRPC progression. (A) Western blot analysis of lysates from LNCaP and LAPC4 prostate cancer cells treated with escalating doses of bicaluatmide for 24 hours. **(B)** Western blot analysis of LNCaP and LAPC4 cell lysates from cultures treated with the indicated doses of anti-androgen for 24 hours. **(C)** Western blot analysis of mTOR activation in LNCaP cells expressing doxycycline inducible shRNA following 72 hours of vehicle or 20μM enzalutamide **(D)** Relative expression levels of CEBPB in RNA isolated from benign prostate, localized prostate cancer and those with heavily treated castrate-resistant prostate cancer. Graph obtained from Oncomine using microarray data from PMID: 22722839. **(E)** NSG mice were subcutaneously engrafted with LNCaP cells harboring shNTV and shCEBPB. When animals had palpable tumors, they were switched to a doxycycline-laced animal feed and surgically castrated one week later. Tumor volume was recorded each week thereafter by caliper measurement. Tumor growth was analyzed by linear regression ($p<0.001$).

The protein kinase known as mTOR is a key driver of protein synthesis in mammalian cells and is hyper-activated in 40-70% of advanced prostate cancers. Because cancer cells grow at much faster rates than ordinary cells, activation of mTOR is necessary to keep up with metabolic demands of the growing tumor. Androgen deprivation therapy, the primary treatment for advanced prostate cancer, was recently suggested to increase mTOR activity and that this event could drive resistance [1]. The findings of these studies reveal that C/EBPβ translational isoforms can be either beneficial or detrimental to prostate cancer cell survival depending on the treatment type. Our studies in androgen sensitive prostate cancer lines show that these cells preferentially express the LIP isoform and that the isoform ratio is maintained when C/EBPβ protein levels are increased during androgen deprivation. Our findings suggest that the higher levels of LIP are critical for maintaining mTOR activation because when we increase LAP expression, mTOR activity decreases. Further, C/EBPβ expression increases in castrate-resistant prostate cancer and suppression of C/EBPβ decreased castrate-resistant prostate cancer tumor

growth. Conversely, LAP expression appeared to be protective when cells were challenged with the proteasome inhibitor, bortezomib. Because bortezomib causes proteotoxicity by accumulation of mis-folded proteins, suppression of protein synthesis by decreasing mTOR activity could promote cell survival in this context. These studies suggest that modulating mTOR activity is a key function of C/EBPβ for adaptation to metabolic stress in prostate cancer cells.

The findings of this research project further our knowledge of the mechanisms that govern protein metabolism in prostate cancer cells and how manipulating C/EBPβ can affect prostate cancer response to androgen deprivation or chemotherapy. Contrary to our expected results, we have found that inhibition of mTOR leads to down-regulation of *CEBPB* transcripts and suppression of all three isoforms in both prostate cancer lines tested. We found that cap-dependent translation was a critical suppressor of LAP isoform expression in prostate cancer cell lines. Another known suppressor of cap-dependent translation, bortezomib, dramatically increased LAP isoforms in LNCaP and PC3 cells. These results may also imply that cap-independent translation is a key driver of LAP-isoforms in PCa cells. Recently, it was demonstrated by two independent groups that under heat-shock stress, cap-independent translation initiation is promoted by *N-6-methylation* of adenosine (m^6A) residues in the 5' UTRs of mRNA transcripts via an increase in YTHDF2, an m^6A "reader" [31, 32]. Interestingly, these groups found that YTHDF2 and HSP70 levels increase beginning at 4 hours following heat shock, which is the same time at which we observe an increase in REDD1 and C/EBPβ protein levels after bortezomib treatment. It is possible that increased m^6A occurs following bortezomib to drive cap-independent translation and LAP isoform expression.

Although clinical trials investigating bortezomib in PCa patients have produced lackluster results, a recent study suggests that the proteasome may still be a valid target in a subset of prostate cancer patients [35]. In patients younger than 65 with localized cancer, proteasome and protein catabolism genes were shown to be strong predictors of metastatic progression after radical prostatectomy. Understanding the response of PCa cells to proteasome inhibition is therefore of potential clinical utility. In the previous reporting period, we found that suppression of C/EBPβ increased sensitivity to bortezomib in PC3 and LNCaP cells and that bortezomib dramatically reduced the growth of PC3 prostate tumors expressing shCEBPB. Because bortezomib is known to trigger autophagy and C/EBPβ can regulate the expression of genes involved in autophagy we tested whether C/EBPβ was involved in regulating autophagy in PCa cells to promote survival. We had intended to generate stable

prostate cancer cell lines with ectopic expression of LAP or LIP isoforms. This proved challenging because C/EBPβ promotes cellular senescence when over-expressed in LNCaP prostate cancer cells, and greatly suppressed the growth of PC3 cells (not shown). We utilized an inducible Tet-on piggyback over expression system to overcome the issues associated with C/EBPβ expression. We took advantage of the fact that LNCaP cells predominantly express LIP and PC3 express higher levels of LAPs to ascertain the function of these isoforms. We found that ectopic C/EBPβ expression differentially regulated autophagy in LNCaP and PC3 cells owing to differences in translational isoform expression. Even though LNCaP cells express higher levels of LIP relative to LAP, knockdown of C/EBPβ still promoted sensitivity to bortezomib, likely because bortezomib rapidly induces LAP isoform expression in these cells.

Our results also demonstrate that C/EBPβ further promotes autophagy through direct induction of *REDD1* gene expression. Previous reports had shown that C/EBPβ cooperates with ATF4 by binding to adjacent half-sites at -1,004 bp in the *REDD1* promoter in cells challenged by oxidative stress [39, 40]. Our ChIP data and over-expression/knockdown studies suggest that C/EBPβ can also bind to a consensus site centered at -603 bp and a near consensus site at -99 bp in the *REDD1* proximal promoter in the absence of oxidative stress to induce *REDD1* expression. REDD1 indirectly promotes autophagosome turnover by inhibiting LC3-II delipidation and thereby preventing LC3 recycling [36]. The latter function is consistent with our findings that show decreased LC3-II and p62 levels and increases in core autophagy regulators in PC3 cells over-expressing C/EBPβ. Because REDD1 is not known to directly mediate or control autophagosome-lysosome fusion, it is likely that there are additional genes regulated by C/EBPβ whose protein products promote this function independent of REDD1. And as noted, shCEBPB expression in PC3 cells only reduced REDD1 2-fold, likely allowing autophagosome formation, albeit at a slower rate. However, we were able to partly rescue the defect in autophagy in shCEBPB cells by ectopic expression of REDD1. In particular, LC3-II was not increased by shCEBPB in the presence of exogenous REDD1, suggesting increased flux through autophagy. The latter effect suggests that in the presence of shCEBPB, where autophagosome-lysosome fusion is suppressed, ectopic expression of REDD1 may have positive feedback on the autophagy pathway or additional activities beyond its ability to induce LC3-II.

Proteasome inhibition increases cytosolic and endoplasmic reticulum (ER) protein content and promotes amino acid deprivation [42, 46]. These events activate the amino acid sensitive protein kinase GCN2 and the unfolded protein response in the ER, leading to phosphorylation of eIF2α and

suppression of protein translation [45]. Autophagy is thought to promote cell survival in cells treated with proteasome inhibitors by restoring amino acid homeostasis and suppressing ER stress [42]. We found that PCa cells treated with bortezomib show a dynamic regulation of autophagy that was dependent upon C/EBPβ expression. Suppression of autolysosome catabolism has been reported in ovarian and breast cancer cell lines treated with bortezomib, owing to a decrease in cathepsins D and B [48, 49]. The conclusions of these studies are consistent with our findings which showed strong up-regulation of *p62* and *ULK1* but suppression of *Cathepsin L* (not shown) in PC3 cells treated with bortezomib.

Our research group discovered that treating prostate cancer cells with androgen deprivation therapy increased the levels of C/EBPβ. Because C/EBPβ was shown to regulate mTOR activity via REDD1 expression, we tested whether suppression of C/EBPβ could decrease CRPC growth or progression. Although mTOR activity was altered as expected in PC3 cells, we did not observe the same effect in LNCaP cells. Oddly, suppression of C/EBPβ decreased the total levels of 4EBP1 and had no effect on p70S6K phosphorylation relative to control cells, indicating that LIP suppresses cap-dependent translation by increasing 4EBP1. Interestingly, we found that suppression of C/EBPβ in LNCaP cells delayed castrate-resistant tumor growth in NOG mice. C/EBPβ is reported to bind within androgen receptor occupied regions (ARORs) in distal enhancer sites of AR-regulated genes and repress transcription [33]. Androgen deprivation is reported to drive the progression of high grade-PIN lesions to invasive carcinoma in a PTEN-loss mouse model of PCa and that this progression is driven by MAPK and AKT signaling [34]. Because AR suppresses AKT signaling, it is possible, that derepression of AR-regulated genes by loss of C/EBPβ could delay CRPC by decreasing AKT and/or MAPK signaling. Future investigations into the role of C/EBPβ in CRPC progression could be directed towards its effects on these signaling pathways and it's regulation of AR-regulated genes.

Progress and Accomplishments:

Key progress made during this reporting period included:

- Completion of Major Task 2. Determined that prostate cancer cell lines lacking C/EBPβ showed increased sensitivity to bortezomib.

- Completion of Major Task 3. Determined that C/EBPβ was critical for CRPC growth in AR-negative PC3 cells and for progression to CRPC in LNCaP cells.

Methods

The methods used for these experiments have been described in the attached publication. Barakat et al., 2015. Statistically significant differences in tumor growth rates were analyzed by linear regression analysis using Graph Pad Prism statistical software.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Ascertained a critical mechanism regulating C/EBPβ translational isoform expression in PCa cells.
- Determined a critical role for C/EBPβ LAP translational isoforms in promoting PCa cell survival via activation of autophagy.
- Demonstrated for the first time that C/EBPβ promotes progression to CRPC and the growth of AR-negative prostate tumors.

5. CONCLUSION:

The results of this research project increased our understanding of how the isoforms of C/EBPβ are regulated, how these isoforms could regulate protein catabolic pathways and cellular growth in prostate cancer cells and how altering the levels of individual C/EBPβ isoforms could effect resistance to chemotherapy or anti-androgens. This work highlights the differential role of C/EBPβ translational isoforms in facilitating autophagy and also raises additional questions into the regulatory mechanisms which guide their translation. The findings of these studies identify C/EBPβ isoforms as novel therapeutic targets in PCa progression to CRPC or in promoting acute toxicity to PCa cells in combination with bortezomib.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Lay Press: none to report.

(2) Peer-Reviewed Scientific Journals:

C/EBPβ regulates sensitivity to bortezomib in prostate cancer cells by inducing REDD1 and autophagosome-lysosome fusion. **Barakat DJ**, Mendonca J, Barberi T, Zhang J, Kachhap SK, Paz-Priel I, Friedman AD. Cancer Lett. 2016 May 28;375(1): 152-61. doi: 10.1016/j.canlet.2016.03.005. PMID: 26968249

CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells. **Barakat DJ**, Zhang J, Barberi T, Denmeade SR, Friedman AD, Paz-Priel I. *Oncogene*. 2015 Mar 16. doi: 10.1038/onc.2015.41. PMID: 25772238

(3) Invited Articles: none to report

(4) Abstracts: none to report

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report.

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

- Attended selected prostate cancer specific research seminars.
- Attended the 2015 American Association for Cancer Research annual meeting.
- Presented ongoing research at Prostate Cancer Research Day (February 2015) and Fellows Research Day (May 2015).
- Attended the 2015 Progenitor Cell Biology Consortium Workshop: CRISPR/Cas9 Genome Engineering for Human Progenitor Cell Biology and Translation. UT Southwestern Medical Center, Dallas, TX (September 2015).
- Publication of results in a peer-reviewed scientific journal (Barakat et al., 2016).
- Submitted revised K99/R00 Pathway to Independence award.Ultimately, this award was not funded. At the next opportunity, I plan to submit a K22 transition award to the NCI.

10. REFERENCES:

1. Carver, B.S., et al., *Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer.* Cancer Cell, 2011. 19(5): p. 575-86.
2. Murillo, H., et al., *Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state.* Endocrinology, 2001. 142(11): p. 4795-805.
3. Ma, X.M. and J. Blenis, *Molecular mechanisms of mTOR-mediated translational control.* Nat Rev Mol Cell Biol, 2009. 10(5): p. 307-18.
4. Zinzalla, V., et al., *Activation of mTORC2 by association with the ribosome.* Cell, 2011. 144(5): p. 757-68.
5. Nakabayashi, M., et al., *Phase II trial of RAD001 and bicalutamide for castration-resistant prostate cancer.* BJU Int, 2012. 110(11): p. 1729-35.
6. Armstrong, A.J., et al., *A pharmacodynamic study of rapamycin in men with intermediate- to high-risk localized prostate cancer.* Clin Cancer Res, 2010. 16(11): p. 3057-66.

7. Paz-Priel, I., et al., *C/EBPalpha or C/EBPalpha oncoproteins regulate the intrinsic and extrinsic apoptotic pathways by direct interaction with NF-kappaB p50 bound to the bcl-2 and FLIP gene promoters.* Leukemia, 2009. 23(2): p. 365-74.

8. Calkhoven, C.F., C. Muller, and A. Leutz, *Translational control of C/EBPalpha and C/EBPbeta isoform expression.* Genes Dev, 2000. 14(15): p. 1920-32.

9. Connors, S.K., et al., *C/EBPbeta-mediated transcriptional regulation of bcl-xl gene expression in human breast epithelial cells in response to cigarette smoke condensate.* Oncogene, 2009. 28(6): p. 921-32.

10. Lin, F.T., et al., *A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity.* Proc Natl Acad Sci U S A, 1993. 90(20): p. 9606-10.

11. Xiong, W., et al., *Regulation of CCAAT/enhancer-binding protein-beta isoform synthesis by alternative translational initiation at multiple AUG start sites.* Nucleic Acids Res, 2001. 29(14): p. 3087-98.

12. Zahnow, C.A., et al., *A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation.* Cancer Res, 2001. 61(1): p. 261-9.

13. Yeh, W.C., et al., *Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins.* Genes Dev, 1995. 9(2): p. 168-81.

15. Bereshchenko, O., et al., *Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPalpha mutant AML.* Cancer Cell, 2009. 16(5): p. 390-400.

16. Gomis, R.R., et al., *C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells.* Cancer Cell, 2006. 10(3): p. 203-14.

17. Cermak, T., et al., *Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting.* Nucleic Acids Res, 2011. 39(12): p. e82.

18. Paz-Priel, I., et al., *CCAAT/enhancer binding protein alpha (C/EBPalpha) and C/EBPalpha myeloid oncoproteins induce bcl-2 via interaction of their basic regions with nuclear factor-kappaB p50.* Mol Cancer Res, 2005. 3(10): p. 585-96.

19. Vinson, C.R., T. Hai, and S.M. Boyd, *Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design.* Genes Dev, 1993. 7(6): p. 1047-58.

20. Wang, S., et al., *Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer.* Cancer Cell, 2003. 4(3): p. 209-21.

21. Cencic, R., Hall, D.R., Robert, F., Du, Y., Min, J., Li, L., Qui, M., Lewis, I., Kurtkaya, S., Dingledine, R., Fu, H., Kozakov, D., Vajda, S. & Pelletier, J., 2011, *Reversing chemoresistance by small molecule inhibition of the translation initiation complex eIF4F.* Proceedings of the National Academy of Sciences of the United States of America, 108(3), pp. 1046-51.

22. Chen, S., Blank, J.L., Peters, T., Liu, X.J., Rappoli, D.M., Pickard, M.D., Menon, S., Yu, J., Driscoll, D.L., Lingaraj, T., Burkhardt, A.L., Chen, W., Garcia, K., Sappal, D.S., Gray, J., Hales, P., Leroy, P.J., Ringeling, J., Rabino, C., Spelman, J.J., Morgenstern, J.P. & Lightcap, E.S., 2010, Genome-wide siRNA screen for modulators of cell death induced by proteasome inhibitor bortezomib, Cancer research, 70(11), pp. 4318-26.

23. Dennis, M.D., Coleman, C.S., Berg, A., Jefferson, L.S. & Kimball, S.R., 2014, REDD1 enhances protein phosphatase 2A-mediated dephosphorylation of Akt to repress mTORC1 signaling, Science signaling, 7(335), p. ra68.

24. Ewing, S.J., Zhu, S., Zhu, F., House, J.S. & Smart, R.C., 2008, C/EBPbeta represses p53 to promote cell survival downstream of DNA damage independent of oncogenic Ras and p19(Arf), Cell death and differentiation, 15(11), pp. 1734-44.

25. Jin, H.O., Seo, S.K., Woo, S.H., Kim, E.S., Lee, H.C., Yoo, D.H., An, S., Choe, T.B., Lee, S.J., Hong, S.I., Rhee, C.H., Kim, J.I. & Park, I.C., 2009, Activating transcription factor 4 and CCAAT/enhancer-binding protein-beta negatively regulate the mammalian target of rapamycin via Redd1 expression in response to oxidative and endoplasmic reticulum stress, Free radical biology & medicine, 46(8), pp. 1158-67.

26. Ramathal, C., Bagchi, I.C. & Bagchi, M.K., 2010, Lack of CCAAT enhancer binding protein beta (C/EBPbeta) in uterine epithelial cells impairs estrogen-induced DNA replication, induces DNA damage response pathways, and promotes apoptosis, Molecular and cellular biology, 30(7), pp. 1607-19.

27. Shirley, C.M., Chen, J., Shamay, M., Li, H., Zahnow, C.A., Hayward, S.D. & Ambinder, R.F., 2011, Bortezomib induction of C/EBPβ mediates Epstein-Barr virus lytic activation in Burkitt lymphoma, Blood, 117(23), pp. 6297-303.

28. Wang L., Shao J., Muhlenkamp P., Liu S., Klepcyk P., Ren J. & Friedman J.E. Increased insulin receptor substrate-1 and enhanced skeletal muscle insulin sensitivity in mice lacking CCAAT/enhancer-binding protein beta. J Biol Chem. 2000 May 12;275(19):14173-81.

29. Liu S, Croniger C, Arizmendi C, Harada-Shiba M, Ren J, Poli V, Hanson RW, Friedman JE. Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBPbeta gene. J Clin Invest. 1999 Jan;103(2):207-13.

30. Ma D. & Lin J.D. Circadian regulation of autophagy rhythm through transcription factor C/EBPβ. Autophagy. 2012 Jan;8(1):124-5. doi: 10.4161/auto.8.1.18081. Epub 2012 Jan 1.

31. Meyer KD, Patil DP, Zhou J, Zinoviev A, Skabkin MA, Elemento O, Pestova TV, Qian SB, Jaffrey SR. 5' UTR m(6)A Promotes Cap-Independent Translation. Cell. 2015 Nov 5;163(4):999-1010. doi: 10.1016/j.cell.2015.10.012.

32. Zhou J, Wan J, Gao X, Zhang X, Jaffrey SR, Qian SB. Dynamic m(6)A mRNA methylation directs translational control of heat shock response. Nature. 2015 Oct 22;526(7574):591-4. doi: 10.1038/nature15377.

33. Jia L, Berman BP, Jariwala U, Yan X, Cogan JP, Walters A, Chen T, Buchanan G, Frenkel B, Coetzee GA. Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. PLoS One. 2008;3(11):e3645. doi: 10.1371/journal.pone.0003645.

34. Jia S, Gao X, Lee SH, Maira SM, Wu X, Stack EC, Signoretti S, Loda M, Zhao JJ, Roberts TM. Opposing effects of androgen deprivation and targeted therapy on prostate cancer prevention. Cancer Discov. 2013 Jan;3(1):44-51. doi: 10.1158/2159-8290.CD-12-0262.

35. Zhao SG, Jackson WC, Kothari V, Schipper MJ, Erho N, Evans JR, Speers C, Hamstra DA, Niknafs YS, Nguyen PL, Schaeffer EM, Ross AE, Den RB, Klein EA, Jenkins RB, Davicioni E, Feng FY. High-throughput transcriptomic analysis nominates proteasomal genes as age-specific biomarkers and therapeutic targets in prostate cancer. Prostate Cancer Prostatic Dis. 18 (2015) 229-236.

36. Qiao S, Dennis M, Song X, Vadysirisack DD, Salunke D, Nash Z, Yang Z, Liesa M, Yoshioka J, Matsuzawa S, Shirihai OS, Lee RT, Reed JC, Ellisen LW. A REDD1/TXNIP pro-oxidant complex regulates ATG4B activity to control stress-induced autophagy and sustain exercise capacity. Nat Commun. 6 (2015) 7014.

37. Chen JJ, Huang WC, Chen CC. Transcriptional regulation of cyclooxygenase-2 in response to proteasome inhibitors involves reactive oxygen species-mediated signaling pathway and recruitment of CCAAT/enhancer-binding protein delta and CREB-binding protein. Mol Biol Cell. 16 (2005) 5579-5591.

38. Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. J Biol Chem. 284 (2009) 12297-12305.

39. Lin L, Stringfield TM, Shi X, Chen Y. Arsenite induces a cell stress-response gene, RTP801, through reactive oxygen species and transcription factors Elk-1 and CCAAT/enhancer-binding protein. Biochem J. 392 (2005) 93-102.

40. Jin HO, Seo SK, Woo SH, Kim ES, Lee HC, Yoo DH, An S, Choe TB, Lee SJ, Hong SI, Rhee CH, Kim JI, Park IC. Activating transcription factor 4 and CCAAT/enhancer-binding protein-beta negatively regulate the mammalian target of rapamycin via Redd1 expression in response to oxidative and endoplasmic reticulum stress. Free Radic Biol Med. 46 (2009) 1158-67.

41. Malagelada C, Ryu EJ, Biswas SC, Jackson-Lewis V, Greene LA. RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. J Neurosci. 26 (2006) 9996-10005.

42. Suraweera A, Münch C, Hanssum A, Bertolotti A. Failure of amino acid homeostasis causes cell death following proteasome inhibition. Mol Cell. 48 (2012) 242-253.

43. Surmacz CA, Pösö AR, Mortimore GE. Regulation of lysosomal fusion during deprivation-induced autophagy in perfused rat liver. Biochemical Journal. 242 (1987) 453-458.

44. Kovacs J, Karpati AP. Regression of autophagic vacuoles in mouse pancreatic cells: a morphometric study of the effect of methylamine and chloroquine followed by cycloheximide treatment. Cell Biol Int Rep. 13 (1989) 805-11.

45. Pizarro-Cerdá J, Desjardins M, Moreno E, Akira S, Gorvel JP. Modulation of endocytosis in nuclear factor IL-6(-/-) macrophages is responsible for a high susceptibility to intracellular bacterial infection. J Immunol. 162 (1999) 3519-3526.

46. Bush KT, Goldberg AL, Nigam SK. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem. 272 (1997) 9086-9092.

47. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends Biochem Sci. 40 (2015) 141-148.

48. Kao C, Chao A, Tsai CL, Chuang WC, Huang WP, Chen GC, Lin CY, Wang TH, Wang HS, Lai CH. Bortezomib enhances cancer cell death by blocking the autophagic flux through stimulating ERK phosphorylation. Cell Death Dis. 5 (2014) e1510.

49. Periyasamy-Thandavan S, Jackson WH, Samaddar JS, Erickson B, Barrett JR, Raney L, Gopal E, Ganapathy V, Hill WD, Bhalla KN, Schoenlein PV. Bortezomib blocks the catabolic process of autophagy via a cathepsin-dependent mechanism, affects endoplasmic reticulum stress and induces caspase-dependent

cell death in antiestrogen-sensitive and resistant ER+ breast cancer cells. Autophagy. 6 (2010) 19-35.

50. Shirley CM, Chen J, Shamay M, Li H, Zahnow CA, Hayward SD, Ambinder RF. Bortezomib induction of C/EBPβ mediates Epstein-Barr virus lytic activation in Burkitt lymphoma. Blood. 117 (2011) 6297-6303.

51. Hungness ES, Robb BW, Luo GJ, Pritts TA, Hershko DD, Hasselgren PO. Proteasome inhibitors activate the transcription factors C/EBP-beta and delta in human intestinal epithelial cells. Biochem Biophys Res Commun. 290 (2002) 469-474.

52. Barakat DJ, Zhang J, Barberi T, Denmeade SR, Friedman AD, Paz-Priel I. CCAAT/enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells. Oncogene. 34 (2015) 5912-5922.

53. Klionsky DJ et al., Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 8 (2012) 445-544.

54. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, Ballabio A. TFEB links autophagy to lysosomal biogenesis. Science. 332 (2011) 1429-1433.

54. Ma D, Panda S, Lin JD. Temporal orchestration of circadian autophagy rhythm by C/EBPβ. EMBO J. 30 (2011) 4642-4651.

55. Guo L, Huang JX, Liu Y, Li X, Zhou SR, Qian SW, Liu Y, Zhu H, Huang HY, Dang YJ, Tang QQ. Transactivation of Atg4b by C/EBPβ promotes autophagy to facilitate adipogenesis. Mol Cell Biol. 33 (2013) 3180-3190.

56. Fels DR, Ye J, Segan AT, Kridel SJ, Spiotto M, Olson M, Koong AC, Koumenis C. Preferential cytotoxicity of bortezomib toward hypoxic tumor cells via overactivation of endoplasmic reticulum stress pathways. Cancer Res. 68 (2008) 9323-9330.

57. Barakat DJ, Mendonca J, Barberi T, Zhang J, Kachhap SK, Paz-Priel I, Friedman AD. C/EBPβ regulates sensitivity to bortezomib in prostate cancer cells by inducing REDD1 and autophagosome-lysosome fusion. Cancer Lett. 2016 May 28;375(1):152-61. doi: 10.1016/j.canlet.2016.03.005. PMID: 26968249

11. APPENDICES:

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Original Articles

C/EBPβ regulates sensitivity to bortezomib in prostate cancer cells by inducing *REDD1* and autophagosome–lysosome fusion

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ABSTRACT

The purpose of this study was to ascertain the mechanisms by which advanced prostate cancer cells resist bortezomib therapy. Several independent studies have shown that cells are protected from proteasome inhibition by increased autophagic activity. We investigated whether C/EBPβ, a transcription factor involved in the control of autophagic gene expression, regulates resistance to proteasome inhibition. In PC3 cells over-expressing C/EBPβ, turnover of autophagic substrates and expression of core autophagy genes were increased. Conversely, C/EBPβ knockdown suppressed autophagosome–lysosome fusion. We also found that C/EBPβ knockdown suppressed *REDD1* expression to delay early autophagy, an effect rescued by exogenous REDD1. Cells with suppressed C/EBPβ levels showed delayed autophagy activation upon bortezomib treatment. Knockdown of C/EBPβ sensitized PC3 cells to bortezomib, and blockade of autophagy by chloroquine did not further increase cell death in cells expressing shRNA targeting C/EBPβ. Lastly, we observed a decreased growth of PC3 cells and xenografts with C/EBPβ knockdown and such xenografts were sensitized to bortezomib treatment. Our results demonstrate that C/EBPβ is a critical effector of autophagy via regulation of autolysosome formation and promotes resistance to proteasome inhibitor treatment by increasing autophagy.

Introduction

Metastatic prostate cancer (PCa) is an incurable disease and a leading cause of cancer death in Europe and North America. When patients acquire resistance to hormonal therapy, second-line chemotherapy is administered, which can increase patient life span by several years. There has been interest in the use of bortezomib, a proteasome inhibitor, for the treatment of PCa, but clinical studies have shown only modest or no significant improvement in patient benefit when administered in combination with hormonal therapy or docetaxel [1–3]. However, a recent study evaluating microarray data from younger patients (<65 years of age) with localized prostate cancer and treated by radical prostatectomy revealed that gene sets representing proteasome subunits and protein catabolism are the strongest predictors of metastatic progression [4]. Thus, proteasome inhibition may still be of clinical value for certain subsets of patients with PCa.

Autophagy is a catabolic process which serves to turn over long-lived proteins and organelles by engulfing them in double-membrane bound vacuoles, autophagosomes. These structures eventually fuse with lysosomes to degrade their contents and recycle lipids, amino acids, and carbohydrates [5]. Autophagy is initiated by activation of a multi-protein type III PI3K complex, composed of Beclin 1, VPS34 and ATG14, which enriches a region of the endoplasmic reticulum (ER) in phosphotidylinositol-3-phosphate to serve as a platform for the recruitment of other proteins to generate an isolation membrane [6,7]. A group of evolutionary conserved proteins are responsible for elongation and closure of the isolation membrane to form a double-membrane autophagosome by proteolytic cleavage of LC3 and GABARAP family proteins, covalently linking them to phosphatidylethanolamine (PE) and incorporating them into the burgeoning autophagosome [8,9]. Autophagy is not only regulated at the initiating steps, as LC3-II can be delipidated by ATG4b to LC3-I to promote LC3 recycling [10]. Conversely, LC3-II delipidation by ATG4b can be suppressed by REDD1, thereby promoting autophagy [11]. Proteins and organelles destined for degradation are trafficked to autophagosomes by the ubiquitin-binding proteins p62 and NBR1 [12–14].

Autophagy has been recognized as a central process that cancer cells utilize to adapt to stress brought about by hypoxia, anoikis, radiation, or chemotherapy. Recently, it has been suggested that

Abbreviations: bZIP, basic region-leucine zipper; C/EBPβ, CCAAT/enhancer binding protein β; ChIP, chromatin immunoprecipitation; CQ, chloroquine; ER, endoplasmic reticulum; GFP, green fluorescent protein; KD, knockdown; NOD/SCID, non-obese diabetic/severe-combined immuno-deficient; NSG, IL2Rγ$^{-/-}$; PE, phosphatidylethanolamine; PCa, prostate cancer; qPCR, quantitative polymerase chain reaction; PB, PiggyBac; mRFP, monomeric red fluorescent protein; shRNA, short hairpin RNA; TSS, transcription start site.

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autophagy plays a critical role in the resistance of PCa cells to hormonal therapy or chemotherapy and that inhibition of autophagy synergizes with docetaxel or androgen deprivation [15,16]. Combined treatment of several different cancer cell lines with autophagy suppressive agents and bortezomib increases cell death and improves clinical response in patients with relapsed multiple myeloma [17–19]. Further, proteasome inhibitor-resistant PCa cells are thought to utilize autophagy to resist this class of drugs [20].

Recently, several groups have shown that autophagy is regulated at the transcriptional level [21,22]. C/EBPβ, a basic region-leucine zipper (bZIP) transcription factor, was identified as a regulator of circadian autophagy in the liver by inducing a broad array of autophagy genes [23]. C/EBPβ protein is translated from an intronless transcript from three in-frame start codons as higher molecular weight, liver-enriched activating proteins (LAP and LAP*) or the truncated liver-enriched inhibitory protein (LIP). The dominant-inhibitory LIP isoform lacks all three N-terminal activation domain modules and can inhibit transcription by heterodimerizing with LAPs. Another group also showed that C/EBPβ promotes differentiation of 3T3-L1 preadipocytes by activation of *ATG4b* gene expression and autophagy [24]. Intriguingly, treatment of Burkitt's lymphoma cells with bortezomib dramatically increases the expression of C/EBPβ [25]. Other proteasome inhibitors, such as lactacystin or MG-132, also increase nuclear levels of C/EBPβ and increase its DNA binding [26,27]. We therefore investigated the role of C/EBPβ in PCa cell autophagy and sensitivity to bortezomib. Our results suggest that autophagy is activated early after bortezomib treatment, that C/EBPβ promotes autophagy in PCa cells via induction of autophagosome–lysosome fusion and via induction of *REDD1*, and that reducing C/EBPβ expression increases prostate cancer cell sensitivity to bortezomib.

Materials and methods

Cell lines, reagents and mice

PC3 and LNCaP cells were maintained in RPMI media with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) supplemented with penicillin/streptomycin. Cells were grown in a humidified incubator maintained at 37 °C with 5% CO_2. Cell lines transduced with short hairpin RNA (shRNA) or PiggyBac (PB) Tet-on vectors were grown in media supplemented with 10% tetracycline-screened FBS (Hyclone). For experiments involving inducible expression of shRNA or ectopic C/EBPβ, 0.5 μg/ml doxycycline was added to the media and replaced every 48 hrs. The pCMS-eGFP-RTP801 plasmid was purchased from Addgene (Cambridge, MA; plasmid #65057).

Non-obese diabetic/severe-combined immuno-deficient (NOD/SCID); IL2Rγ$^{-/-}$ (NSG) mice were obtained from a breeding colony established in the Johns Hopkins University division of animal resources. The animals were subcutaneously engrafted with 2E06 PC3 cells in PBS in a 1:1 mixture with Matrigel. The animals were monitored daily after transplantation. When tumor volumes reached 100 mm^3, the mice were placed on doxycycline-laced feed. When tumors reached a volume between 100 and 300 mm^3, the animals were randomly assigned to receive either DMSO vehicle or bortezomib, by intraperitoneal (IP) injection (1 mg/kg of body weight) on days 1, 4 and 8. The mice were sacrificed 11 days after engraftment. Tumor volumes were determined by caliper measurement using the ellipsoidal formula: length × width × height × 0.5236 [28].

All animal studies were conducted under protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western blotting

Protein samples from whole cell lysates or nuclear extracts were prepared and subjected to Western blotting as described [29]. Each experiment was repeated at least twice. Anti-β-actin (AC15) and anti-vinculin (V9131) antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-C/EBPβ (C-19), anti-C/EBPβ (H-7), and anti-SQSTM1/p62 (H-290) antibodies were from Santa Cruz Biotechnologies (Dallas, TX). Anti-LC3B (2775), anti-ATG3 (3415), anti-ATG5 (8540), and anti-ATG-7 (8558) antibodies were from Cell Signaling Technologies (Danvers, MA). Anti-REDD1 antibody (10638-1-AP) was from Proteintech (Rosemont, IL).

Quantitative real-time PCR

RNA extraction, cDNA synthesis, and PCR reactions were performed as previously described [29]. Oligonucleotides were designed using PrimerBlast and custom ordered from Sigma-Aldrich. Their sequences are presented in Table 1.

Table 1
PCR primers.

Gene	Forward (5′–3′)	Reverse (5′–3′)
ACTB	GACCTGGCTGGCCGGGACCT	GGCCATCTCTTGCTCGAAGT
ATG4B	ATGAGCATCGCGGAGCTTG	CTAGGGACAGGTTCAGGACG
ATG5	GAAGCTGTTTCGTCCTGTGG	GATGTTCACTCAGCCACTGC
ATG7	ACAGCTTGTTCTTCCAAAGTTCT	TCTCAGATGGTCTCATCATCGC
CTSL	CCTCAGCATGAGGAACACGA	AAAAGGTGCTGGTGGAGGTT
BNIP3	CCTCAGCATGAGGAACACGA	AAAAGGTGCTGGTGGAGGTT
BNIP3L	TGGAGCCATGAAGAAAGGGG	ACTTCACAGGTCACACGCAT
GABARAP	GGCTCCCAAAGCTCGGATAG	CTGGTACAGCTGACCCATTGT
REDD1	CGAACTCCCACCCCAGATCG	AACGACACCCCATCCAGGTA
p62	GATTCGCCGCTTCAGCTTCTG	GTCACTGGAAAAGGCAACCAA
ULK1	CGCCATCTCCTCAAGTTGGA	TGCAAGTCAGACAGGTTGGG

Ectopic C/EBPβ expression and shRNA

LNCaP cells stably transfected with the TET-on, transposon based PiggyBac vector PB-TRE, or PB-CEBPB expressing murine C/EBPβ, were previously described [29]. PC3 cells were transfected with these same vectors, and cells with stable integration were positively selected with puromycin (2 mg/ml). Lentiviral transduction of PC3 cells with pTRIPZ lentiviral vectors targeting *CEBPB* was also performed as previously described for LNCaP cells [29].

TALEN construction and CEBPB gene editing

TALEN DNA constructs targeting the human *CEBPB* open reading frame (ORF) were constructed as previously described [29]. CEBPB homology arm 1 (HA1) was amplified from 293T DNA with primers TGCtctagaCTGGTGGGAACAATGCCACC and ACTtgatcaTGGTGGCATTGTTCCCACCAG; restriction enzyme sites are in lower case. The resulting fragment was digested with *Xba*I and *Spe*I and ligated into the pSEPT donor plasmid [30]. CEBPB homology arm 2 (HA2) was amplified using GCatcgatGAACTTGTTCAAGCAGCTGCC and AGTcagctgAGGCTCCGGAATCTCTTCTC primers. The resulting fragment was digested with *Cla*I and *Sal*I and ligated into the pSEPT plasmid containing CEBPB HA1. PC3 cells were co-transfected with TALEN expression vectors targeting *CEBPB* and the pSEPT donor plasmid at a 1:1 ratio and after 48 hours were seeded into a 96-well plate at 1 cell/well with G418. Individual clones were screened for C/EBPβ expression by Western blotting.

Chromatin immunoprecipitation (ChIP)

1E06 PC3 cells were used in each ChIP reaction, using anti-rabbit C/EBPβ antiserum or normal rabbit IgG (Santa Cruz Biotechnology) as previously described [31]. DNA fragments corresponding to the promoter of *REDD1* were detected by qPCR using the primers presented in Table 2.

Cell viability and proliferation assays

To assess cell proliferation, 1E05 PC3 cells were seeded in growth media containing 0.5 μg/ml doxycycline. On days 2 and 4, cells were trypsinized and stained with Trypan blue dye, and viable cells were enumerated using a hemocytometer. Experiments were repeated three times. For clonogenic assays, PC3 cells were trypsinized and seeded into 6-well plates at 200 cells per well. 12 days after plating, cells were fixed and stained with 4% formaldehyde and 0.05% crystal violet. After washing with tap water, colonies were manually counted under a bright field microscope. Clusters of cells containing more than 50 cells were counted as a colony.

Autophagosome–lysosome fusion assay

PC3 cells were plated on 60 mm dishes to obtain 80% confluence on the next day. Cells were transfected with tandem mRFP-GFP fluorescent-tagged LC3 (ptfLC3) (Addgene #21074) [32]. On day two post-transfection, cells were trypsinized and plated on sterile glass bottom dishes (Mattek, Ashland, MA) at a 40–50% confluency.

Table 2
ChIP primers.

Region	Forward (5′–3′)	Reverse (5′–3′)
REDD1 [–198 : –17]	GGGTTCGACTGCTGCGAGCTTTC	AGCAGCCTATAAGGACTAGCG
REDD1 [–720 : –534]	CTCTCCTTGGGAGCAGTTGG	TGCAAAGGCTCGGGATATGG

Cells were allowed to adhere to glass and spread for 24 hrs. Cells were then imaged using a live cell Zeiss LSM780-FCS Single-point, laser scanning confocal microscope at the JHU Core Imaging facility. Images were processed using ImageJ software. Cells with predominantly yellow RFP/GFP (autophagosome) or red RFP (autolysosome) punctae were counted and analyzed as described [32].

Q74-EGFP degradation assay

PC3 cells seeded in 24-well plates were transfected with 500 ng of Q74-EGFP (Addgene #40262) [33] or pMax-GFP (Lonza, Basel, Switzerland). Forty-eight hours after transfection, fluorescent micrographs of GFP-positive cells were taken using a Nikon Ti-E inverted microscope at 400× magnification from at least five non-overlapping regions of interest. Cells were manually counted using ImageJ software and the percentage of GFP-positive cells displaying punctuate staining was determined.

Statistical analysis

Statistical comparison of the two groups was conducted using Student's t-test. Comparisons of multiple groups were performed using analysis of variance followed by multiple comparisons with Student's t-test and the Holm–Bonferonni correction. P-values were ordered from highest to lowest and α was adjusted to $\alpha/(m+1-k)$ for $p_{(k)}$, where m = number of comparisons and corresponds to the highest p-value. k corresponds to the p-value rank. α was set to 0.05 for comparisons. For analysis of tumor growth, linear regression analysis was used to determine statistical significance in growth rates between groups of mice using Graph Pad Prism statistical software.

Results

C/EBPβ LAP : LIP isoform ratio regulates autophagy in prostate cancer cells

To understand the role of C/EBPβ in prostate cancer cell autophagy, we first ectopically expressed murine C/EBPβ, which shares 98.8% similarity to the human C/EBPβbZIP domain and 80–100% similarity to the human trans-activation and regulatory domains, in the PC3 PCa cell lines using the PB-TRE Tet-on PiggyBac inducible expression vector we had used previously to obtain LNCaP cells harboring doxycycline-inducible C/EBPβ [29]. Induction of PiggyBac-CEBPB (PB-CEBPB) in PC3 cells led to a decline in the total levels of LC3-I and LC3-II and p62 compared to cells expressing the empty PB-TRE vector (Fig. 1A, left). Decreases in p62 levels typically indicate increased autophagic degradation, while changes in LC3-II can imply blockade or activation of autophagosome formation [34]. We further evaluated other proteins involved in autophagosome nucleation and elongation and found increases in ATG3, ATG5, ATG7 and Beclin-1, suggesting that autophagy is increased upon induction of C/EBPβ (Fig. 1A, right).

In contrast with these results in PC3 cells, LNCaP cells showed increased total levels of LC3-I and LC3-II, and no change in p62 in response to C/EBPβ induction (Fig. 1B). Of note, ectopic C/EBPβ is predominantly expressed as the shorter, dominant inhibitory LIP isoform in LNCaP cells as is endogenous C/EBPβ. Thus induction of CEBPB in LNCaP cells may reduce C/EBPβ trans-activation activity to impair autophagy.

As C/EBPβ was shown to directly regulate autophagic genes at the transcriptional level [23,24,35], we also evaluated the expression of several such genes by quantitative real-time PCR (qPCR). We found a modest increase in *CTSL*, *GABARAP*, *ATG7*, *BNIP3*, *BNIP3L* and *ULK1* mRNAs in PC3 cells and nearly all of these genes were suppressed upon ectopic C/EBPβ expression in LNCaP cells (Fig. 1C).

C/EBPβ promotes autophagosome–lysosome fusion in PC3 cells

Bafilomycin A1, a vacuolar-ATPase inhibitor, increases lysosomal pH and alters Ca^{2+} gradients of the lysosome, subsequently

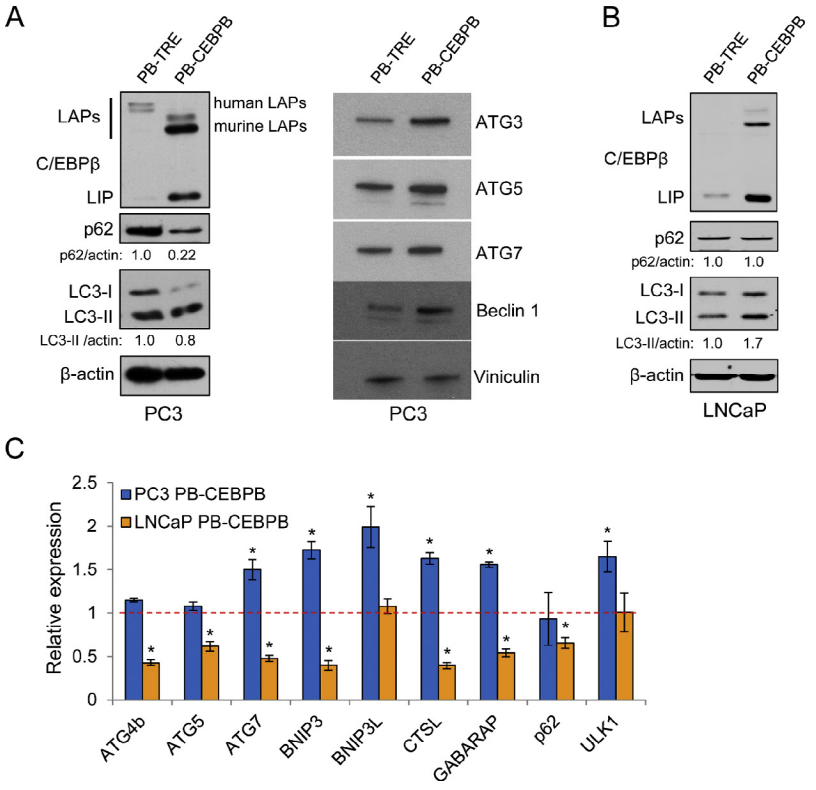

Fig. 1. C/EBPβ isoforms LAP and LIP differentially regulate autophagy in PCa cells. (A) PC3 cells stably transduced with PB-TRE or PB-CEBPB were treated for 72 hrs with 0.5 μg/ml doxycycline and cell lysates were analyzed by Western blotting for the indicated proteins. Numbers below the blots indicate the relative band density normalized to β-actin. (B) LNCaP cells stably transduced with PB-TRE or PB-CEBPB were analyzed similarly for C/EBPβ, p62, LC3, and β-actin. (C) PC3 or LNCaP cells transduced with PB-TRE or PB-CEBPB were cultured similarly followed by qPCR analysis for the indicated genes. The dashed line represents gene expression levels in control cell lines expressing PB-TRE. Bar graphs represent the average of three experiments, error bars represent standard error of the mean (SEM) (*$p < 0.05$).

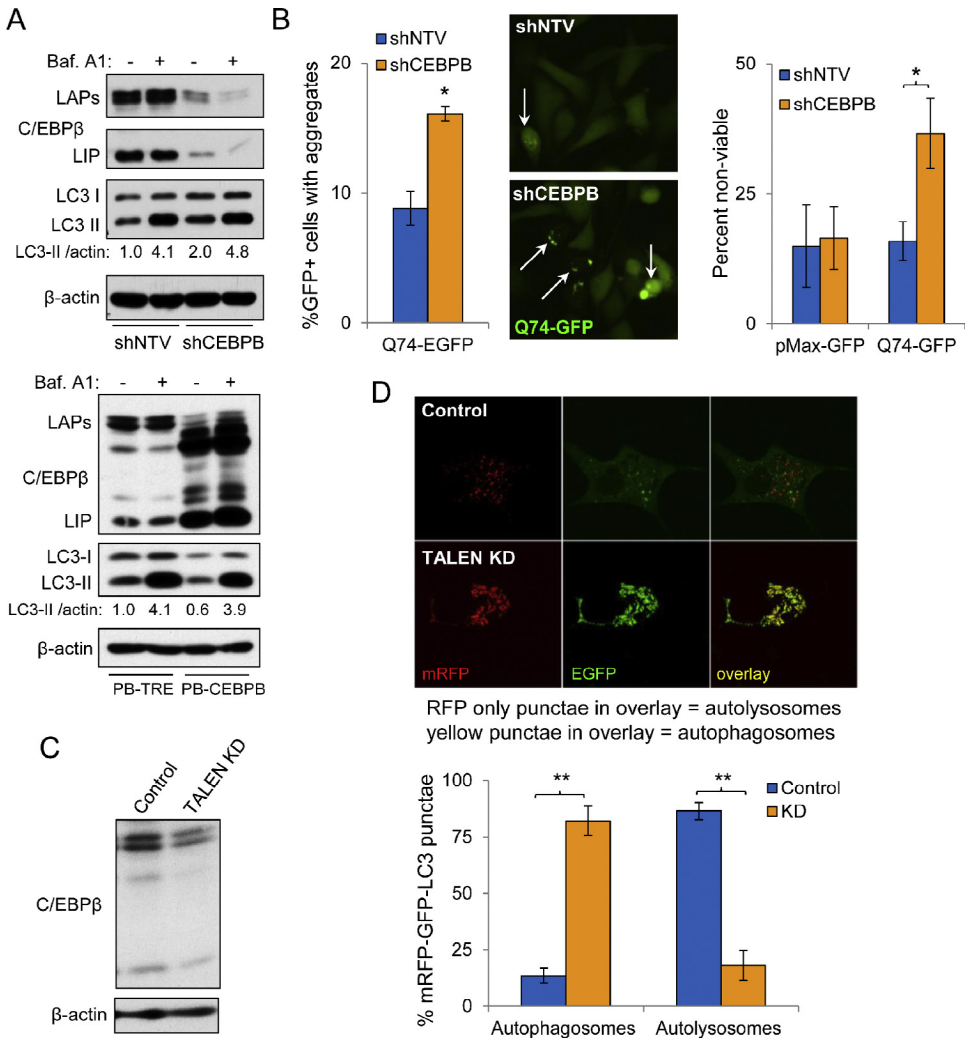

Fig. 2. C/EBPβ promotes autophagosome–lysosome fusion in PC3 cells. (A) Western blot analyses in PC3 cells expressing NTV or CEBPB shRNAs (top panels) or ectopically expressing mouse C/EBPβ from a PiggyBac vector (PB-CEBPB) or the PB-TRE control vector (bottom panels). In both sets of experiments, PC3 cells were treated for 72 hrs with 0.5 μg/ml doxycycline and then treated with either vehicle (DMSO) or 100 nM bafilomycin A1 for 4 hrs. Western blotting for C/EBPβ, LC3, and β-actin was then conducted. (B) Quantification of EGFP-Q74 aggregates in GFP+, transfected PC3 cells expressing shNTV or shCEBPB (left). Sample fluorescence micrograph (center); white arrows indicate cells with aggregates. Cell viability was quantified by Trypan blue dye exclusion in cultures transfected with control pMax-GFP or EGFP-Q74 (right). (C) Western blot analysis of cell lysates from PC3 control and TALEN KD subclones. (D) Confocal micrographs of PC3 TALEN control and CEBPB KD cells transfected with the tandem fluorescent-tagged mRFP-GFP-LC3 (tfLC3) construct (top panels). Quantification of percent of cells with LC3 present in autophagosomes or autolysosomes is shown below (bottom). In the red/green overlay images, yellow foci represent autophagosomes and red foci autolysosomes. Bar graphs throughout represent the average of three experiments, error bars represent SEM (*$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inhibiting fusion with autophagosomes, blocking autophagic flux and leading to accumulation of LC3-I and LC3-II [36,37]. We treated cells for four hours with bafilomycin A1 and then evaluated lysates by Western blot analysis. Although the total levels of LC3-I and LC3-II were increased in PC3 cells expressing shCEBPB, the ultimate level of LC3-II achieved in response to bafilomycin A1 was not different from the control cells transduced with shNTV (Fig. 2A, top). In addition, ectopic expression of C/EBPβ did not enhance autophagic flux in bafilomycin A1-treated cells, as again the peak levels of LC3-II, 4.1 and 3.9, were nearly identical (Fig. 2A, bottom). These data suggest that C/EBPβ does not play a dominant role in autophagosome formation, as otherwise CEBPB shRNA might have been predicted to reduce and ectopic C/EBPβ to increase the change in LC3-II in the presence of bafilomycin A1.

We next tested whether C/EBPβ regulates turnover of autophagy substrates. We transiently expressed mutant huntingtin-EGFP (Q74-EGFP) fusion protein in PC3 cells together with either shNTV or shCEBPB [33]. Expression of Q74-EGFP results in protein aggregation, which can be observed as punctuate GFP-positive staining. Forty-eight hours after transfection, we quantified the number of GFP-positive cells displaying aggregates and found that there were significantly more cells with GFP aggregates in PC3 cells expressing shRNA targeting CEBPB (Fig. 2B, left and center). These data indicate that turnover of autophagosomes is augmented by C/EBPβ. In addition, there were significantly more non-viable, Trypan blue dye positive cells in cultures lacking C/EBPβ (Fig. 2B, right).

The first step in autophagosome turnover is fusion with lysosomes. We therefore next evaluated autophagosome–lysosome fusion in PC3 cells using plasmid ptfLC3, expressing a mRFP-GFP-LC3 fusion protein [32]. As GFP fluorescence is quenched by the acidic environment of the lysosome, yellow punctae visible under fluorescence microscopy, representing signal from both RFP and GFP, indicate autophagosomes, whereas red punctae represent autolysosomes. Doxycycline induction of shRNA from pTRIPZ also induces RFP and so is not amenable to this assay. We therefore utilized TALEN targeting of genomic *CEBPB* as an alternative means of knockdown (KD).

We utilized homologous recombination to insert a neomycin resistance cassette and generate a deletion in the *CEBPB* locus with a pSEPT donor plasmid containing homology arms of CEBPB flanking the TALEN cut site (Supplementary Figure S1) [30]. We generated a neomycin-resistant subclone which expressed decreased levels of C/EBPβ relative to the control subclone (Fig. 2C). When TALEN KD cells were transfected with the mRFP-GFP-LC3 reporter, we found that there was a remarkable, 4-fold increase in the percentage of cells with RFP/GFP-positive autophagosomes relative to control cells and a similar decrease in the percent of cells with RFP-positive autolysosomes, implying that fusion with lysosomes is impaired (Fig. 2D). Collectively, these data demonstrate that C/EBPβ is critical for basal autophagy in PC3 cells by regulating autophagosome–lysosome fusion.

C/EBPβ LAP promotes autophagy in PC3 cells by augmenting REDD1 expression

Although C/EBPβ is reported to broadly regulate the expression of autophagy genes in the liver [23], we did not observe differential expression of all genes analyzed in PC3 cells overexpressing C/EBPβ. Recently, it was shown that REDD1 could promote autophagy by suppressing ATG4b-mediated LC3-II delipidation to LC3-I, an effect most critical during periods of metabolic stress [11]. This function of REDD1 prevents recycling of LC3-II and leads to greater turnover of autophagosomes. REDD1 can also inhibit mTORC1, which suppresses autophagy by phosphorylating ULK1 and ATG13, upstream autophagy regulators [38]. C/EBPβ was previously shown to cooperate with ATF4 to promote *REDD1* transcription by binding to a non-consensus site centered 1004 bp upstream from the *REDD1* transcription start site (TSS), but only in cells under oxidative stress [39,40]. We evaluated *REDD1* gene expression changes in PCa cells expressing shRNA targeting *CEBPB* by qPCR. In contrast to cells overexpressing C/EBPβ, we found that none of the genes that we had analyzed in Fig. 1 were affected by C/EBPβ KD in LNCaP or PC3 cells (Fig. 3A). However, in PC3 cells with *CEBPB* deficiency, we found a significant 2-fold down-regulation of *REDD1* transcript levels (Fig. 3A). We also found that LNCaP cells showed increased *REDD1* upon shCEBPB induction, likely due to decreased expression of the dominant-inhibitory LIP isoform (Fig. 3A). Notably, *REDD1* mRNA showed stronger up-regulation upon C/EBPβ over-expression in PC3 cells than other autophagy genes analyzed (Fig. 3B). Conversely, overexpression of C/EBPβ in LNCaP cells suppressed *REDD1* transcript levels (Fig. 3B).

As we observed that *REDD1* expression correlated with full-length C/EBPβ levels in the absence of oxidative stress, we analyzed the human *REDD1* promoter to identify additional binding sites for C/EBPβ. We found a site matching the C/EBPβ consensus at –603 bp and a near-consensus site at –99 bp (Supplementary Figure S2). To test whether C/EBPβ binds to these regions, we subjected nuclear extracts from PC3 cells to chromatin immunoprecipitation (ChIP). Using two different primer pairs, each flanking one of the putative C/EBPβ binding sites, we observed between 6 and 8-fold greater levels of amplified product in extracts precipitated with anti-C/EBPβ antiserum relative to normal rabbit IgG (Fig. 3C), indicating that endogenous C/EBPβ binds to these regions of the *REDD1* promoter.

C/EBPβ regulation of REDD1 suggests that C/EBPβ not only is critical for autophagosome–lysosome fusion, but also might play a role in autophagosome maturation. Residual REDD1 present upon shCEBPB-mediated *CEBPB* KD must be sufficient to prevent a complete block at this step in autophagy. Knockdown of REDD1 was problematic, as all siRNAs tested also reduced C/EBPβ (not shown), and of note REDD1(–/–) MEFs show greatly reduced autophagosome

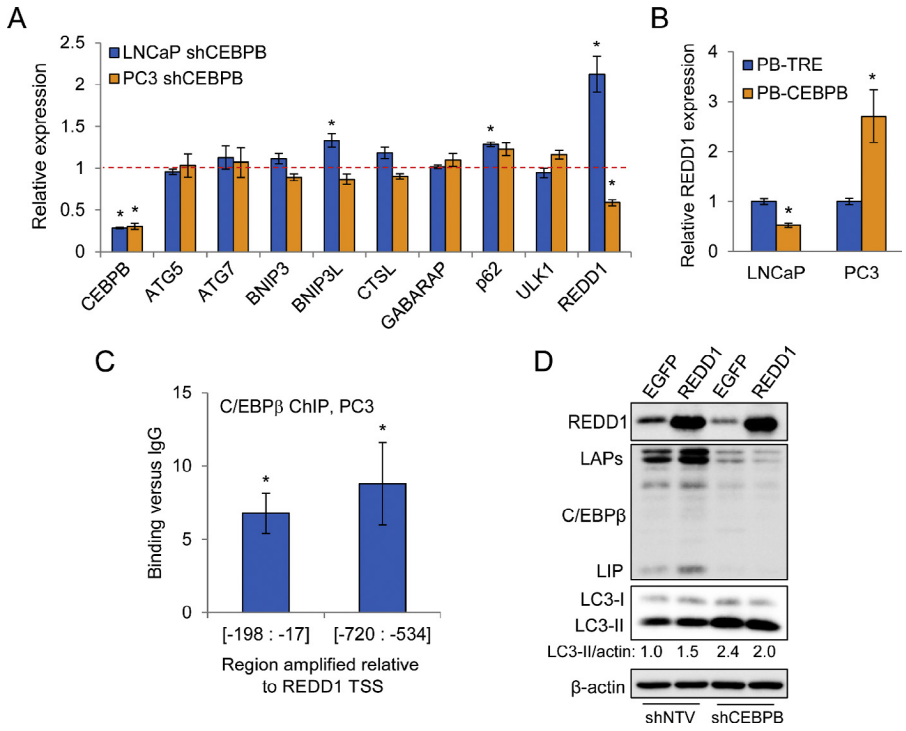

Fig. 3. C/EBPβ promotes autophagy in PC3 cells by regulation of *REDD1* expression. (A) qPCR analysis of autophagic genes in PC3 and LNCaP cells expressing shRNA targeting *CEBPB*. The dashed line represents gene expression levels in control cell lines expressing shNTV. (B) *REDD1* gene expression in cells expressing C/EBPβ from the PB-CEBPB vector or harboring the control PB-TRE vector after 72 hr culture with doxycycline. (C) qPCR analysis, using two primer pairs, of genomic DNA extracted from PC3 cell chromatin following immunoprecipitation with anti-C/EBPβ antibody or IgG control. Fold-increase in C/EBPβ binding versus IgG is shown. (D) PC3 cells expressing shNTV or shCEBPB were transiently transfected with pMax-GFP or pCMS-EGFP-REDD1, and 48 hours later whole cell lysates were subject to Western blot analysis for the indicated proteins. Data presented are representative of three independent experiments. Bar graphs represent the average of three experiments, error bars represent SEM (*$p < 0.05$).

formation [11]. Therefore, to further test whether C/EBPβ drives early autophagy via *REDD1*, we transiently over-expressed REDD1 in PC3 cells. We found that expression of REDD1 from the pCMS-EGFP-REDD1 vector [41] increased LC3-II in PC3 cells harboring shNTV, as predicted from its role in early autophagy, but mildly reduced LC3-II in shCEBPB cells, compared to controls expressing the pMax-GFP plasmid (Fig. 3D). These results suggest that C/EBPβ promotes autophagy in part by regulating *REDD1* expression.

C/EBPβ promotes autophagy following bortezomib treatment

Previous reports suggest that proteasome inhibitors increase expression and activity of C/EBPβ [25,26]. As proteasome inhibitors also activate autophagy [42,43], we tested whether C/EBPβ is required for autophagy following bortezomib treatment. Gene expression analysis of several autophagy genes revealed that *ULK1* and *p62* were markedly increased 16 hours after bortezomib treatment of PC3 cells (Fig. 4A). *ATG5* and *ATG4B* were unchanged and *ATG7* and *REDD1* were mildly upregulated by bortezomib. The changes in *p62* and *ULK1* were not mediated by C/EBPβ because shCEBPB cells showed the same increase in gene expression upon bortezomib treatment (Fig. 4B). Treatment of LNCaP or PC3 cells with bortezomib for 24 hours markedly increased C/EBPβ (LAP), LC3-II, and p62 protein expression (Fig. 4C). To further evaluate the effects of bortezomib on PCa cell autophagy, we analyzed LC3 and p62 by time course analysis. We found that REDD1 increased from 4 to 8 hours following 25 nM bortezomib treatment, but then decreased between 8 and 16 hours (Fig. 4D). LC3-II and p62 protein levels showed the strongest increase 16 hours after treatment. Lastly, we tested whether C/EBPβ was critical for autophagy following bortezomib exposure. We treated PC3 cells expressing shCEBPB with bortezomib and evaluated autophagy markers by time course analysis. C/EBPβ deficiency decreased REDD1 expression and prevented increases in LC3-II, though shCEBPB cells had higher basal levels of LC3-II (Fig. 4E). These data indicate that bortezomib promotes autophagy and that C/EBPβ is critical for activating autophagy early after bortezomib treatment.

Reduced C/EBPβ slows PC3 tumor growth and increases sensitivity to bortezomib

Autophagy positively regulates "cellular fitness" in a variety of cell types [44]. We therefore evaluated the effect of C/EBPβ on PC3 proliferation. We quantified cell growth over a four day time course and found that inhibition of C/EBPβ by shRNA targeting or TALEN reduced cell accumulation by more than 2-fold (Fig. 5A). We also evaluated clonogenic growth and found that cells deficient in C/EBPβ due to TALEN targeting or shRNA expression showed a more than two-fold average decrease in colony number (Fig. 5B). We treated shNTV and shCEBPB PC3 cells with 25 or 50 nM of bortezomib for 24 hours and evaluated viability by Trypan blue dye exclusion. Suppression of C/EBPβ increased cell death relative to shNTV cells by 2-fold with either dose of bortezomib (Fig. 5C, left). Phase contrast images of cells treated with 50 nM bortezomib and shNTV or shCEBPB are also shown (Fig. 5C, right). To evaluate whether autophagy contributed to increased cell death in shCEBPB cells, we treated cells with chloroquine (CQ) in combination with bortezomib. Chloroquine is a weak base that accumulates in the acidic compartments of late endosomes and lysosomes, alkalinizes these structures, and subsequently inhibits autophagosome–lysosome fusion [45,46]. We found that there was no difference in shNTV versus shCEBPB cell death in cultures treated with the combination of CQ and bortezomib, indicating that increased death in shCEBPB cultures was due to a block in autophagy (Fig. 5D). We next

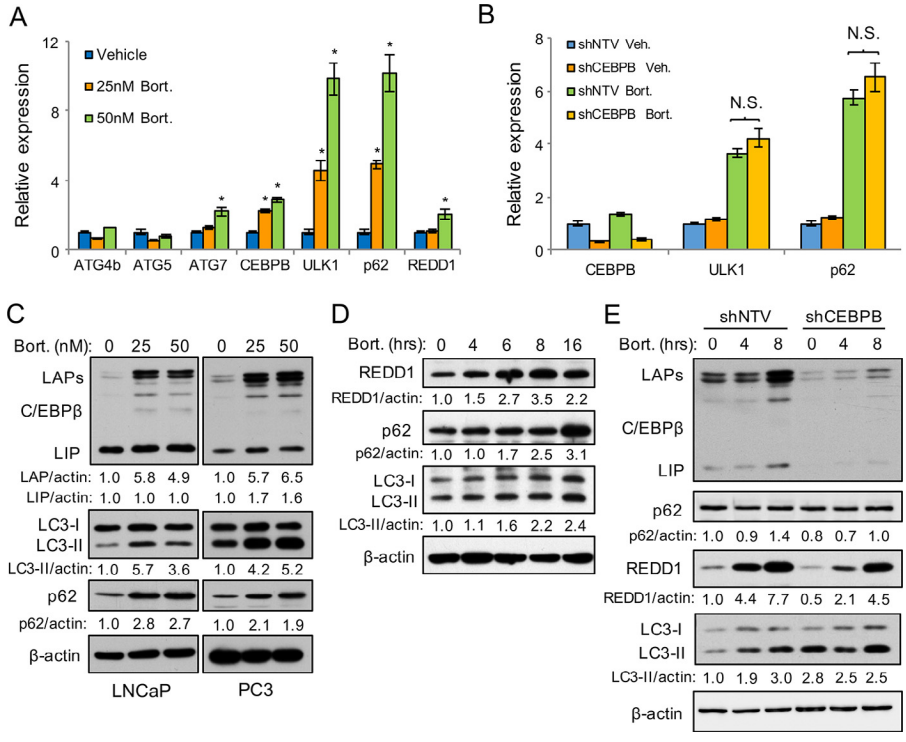

Fig. 4. C/EBPβ promotes autophagy driven by bortezomib challenge. (A) qPCR analysis for indicated mRNAs from PC3 cells that had been treated with bortezomib or vehicle for 16 hrs. (B) qPCR analysis of autophagy genes in PC3 cells expressing shNTV or shCEBPB and that had been treated for 16 hrs with 25 nM bortezomib or vehicle. (C) LNCaP or PC3 parental cells were treated for 24 hrs with the indicated concentrations of bortezomib and cell lysates were then subjected to Western blot analysis. (D) PC3 cells were treated with 25 nM bortezomib for the indicated times and cell lysates were then analyzed by Western blot analysis for indicated proteins. (E) PC3 cells expressing NTV or CEBPB shRNAs were treated with 50 nM bortezomib for the indicated times and cell lysates were again analyzed by Western blot analysis. Numbers below blots indicate relative density after normalizing to β-actin. Bar graphs represent the average of three experiments, error bars represent SEM (*$p < 0.05$).

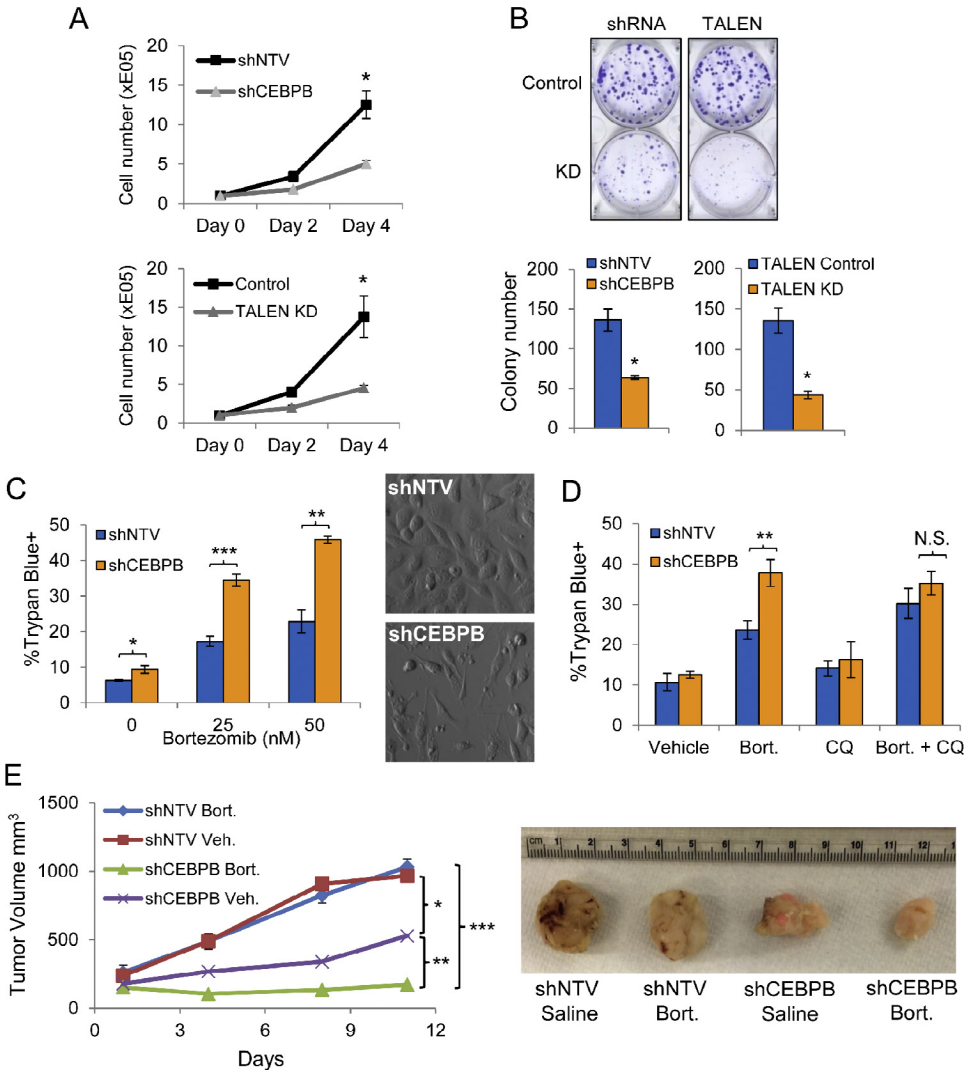

Fig. 5. C/EBPβ deficiency suppresses PC3 growth and improves the *in vivo* efficacy of bortezomib. (A) Growth of indicated PC3 lines after 3 and 5 days of culture (n = 3). (B) Quantification of clonogenic growth of the indicated PC3 lines 12 days after seeding in 6-well plates at 200 cells/well. Crystal violet staining of representative wells (top) and mean colony numbers (bottom) are shown. (C) PC3 cells were incubated for 72 hrs in 0.5 μg/ml doxycycline prior to treatment with the indicated concentrations of bortezomib for 24 hrs. Quantification of cell viability by Trypan blue dye exclusion is shown (left). Representative phase contrast images show shNTV and shCEBPB PC3 cells that had been treated with 50 nM bortezomib for 24 hrs (right). (D) Quantification of cell viability by Trypan blue dye exclusion in PC3 cultures that had been treated with the indicated drugs for 24 hrs. Bort – 25 nM bortezomib; CQ – 50 uM chloroquine. (E) Quantification of PC3 tumor growth in NSG mice treated with vehicle or bortezomib (1 mg/kg) on days 1, 4 and 8. (n = 7). Image to the right of the figure shows representative tumors from the indicated groups. Statistically significant differences were determined by linear regression analysis. Alpha adjusted by Holm–Bonferroni correction. Bar graphs represent the average of three experiments, error bars represent SEM (*p < 0.05; **p < 0.025; ***p < 0.0167).

evaluated PC3 tumor growth and sensitivity to bortezomib *in vivo*. NSG mice subcutaneously engrafted with shNTV or shCEBPB PC3 cells were treated with either vehicle or bortezomib (1 mg/kg) on days 1, 4 and 8 (Fig. 5E). Consistent with our *in vitro* findings, we observed a decreased rate of growth of shCEBPB PC3 tumors (p = 0.026). Further, we observed no difference in the rate of growth in control tumors receiving either vehicle or bortezomib. In contrast, bortezomib treatment and *CEBPB* KD strikingly synergized to further decrease tumor growth (p = 0.017). These results suggest that C/EBPβ is critical for prostate tumor growth and that decreasing C/EBPβ increases sensitivity to bortezomib *in vivo*.

Discussion

Although clinical trials investigating bortezomib in PCa have produced lackluster results, a recent study suggests that the proteasome may still be a valid target in a subset of prostate cancer patients [4]. In patients younger than 65 with localized cancer, proteasome and protein catabolism genes were shown to be strong predictors of metastatic progression after radical prostatectomy. Understanding the response of PCa cells to proteasome inhibition is therefore of potential clinical utility.

The results of our study suggest that C/EBPβ regulates fusion of lysosomes and autophagosomes in PCa cells. The effects of C/EBPβ on autophagy were dependent upon the expression ratio of the C/EBPβ translational isoforms, with higher relative levels of LIP : LAP expression suppressing autophagy in LNCaP cells and vice versa in PC3 cells. The observed effect of LIP down-regulating autophagic gene expression is consistent with its role as a transcriptional repressor owing to its lack of N-terminal trans-activation domains. Our data are consistent with previous reports that show a link between C/EBPβ and autophagy. C/EBPβ was reported to control circadian autophagy in mouse liver by broadly activating autophagy gene transcription [23]. Guo and colleagues showed that C/EBPβ activates

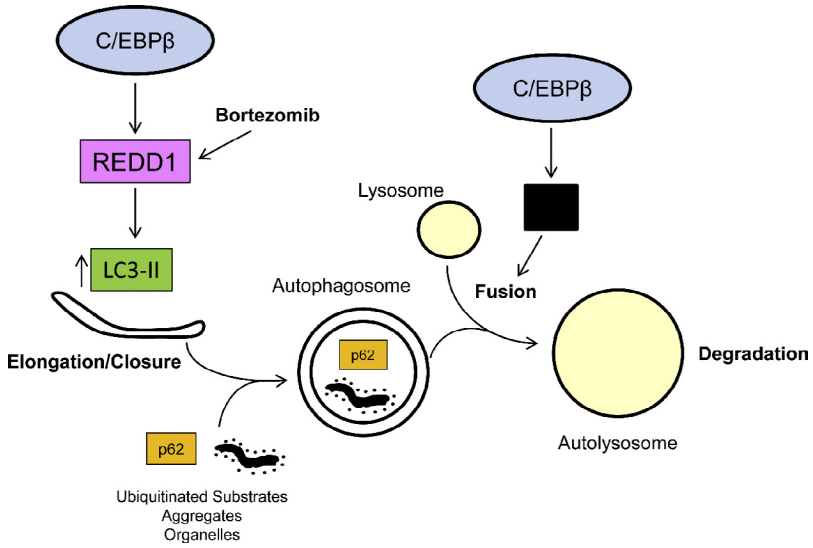

Fig. 6. Model for C/EBPβ driven autophagy in PCa cells. C/EBPβ promotes autophagy in PCa cells by promoting autophagosome–lysosome fusion and by driving *REDD1* gene expression. REDD1 suppresses LC3-II delipidation to LC3-I to increase autophagosome formation. Bortezomib may promote autophagy by increasing REDD1 protein levels. In addition, autophagosome–lysosome fusion is directly driven by C/EBPβ through an as of yet undescribed mechanism (indicated by the black box).

autophagy by directly regulating *ATG4B* in 3T3-L1 pre-adipocytes to promote differentiation to mature adipocytes [24]. However, the specific defect in the autophagosome–lysosome fusion that we have revealed in PCa cells is a distinct mechanism from that reported in these earlier studies (Fig. 6). Analogous to what we have found, however, C/EBPβ(–/–) macrophages exhibit defects in bactericidal activity and fusion of phagosomes with lysosomes [47]. Overall, our findings and those of others show that the effect of C/EBPβ on autophagy is cell type and context specific.

Our results also demonstrate that C/EBPβ further promotes autophagy through direct induction of *REDD1* gene expression (Fig. 6). Previous reports had shown that C/EBPβ cooperates with ATF4 by binding to adjacent half-sites at –1004 bp in the *REDD1* promoter in cells challenged by oxidative stress [39,40]. Our ChIP data and over-expression/knockdown studies suggest that C/EBPβ can also bind to a consensus site centered at –603 bp and a near consensus site at –99 bp in the *REDD1* proximal promoter in the absence of oxidative stress to induce *REDD1* expression. REDD1 indirectly promotes autophagosome turnover by inhibiting LC3-II delipidation, thereby preventing LC3 recycling [11]. The latter function is consistent with our findings that show decreased LC3-II and p62 levels and increases in core autophagy regulators in PC3 cells over-expressing C/EBPβ. Because REDD1 is not known to directly mediate or control autophagosome–lysosome fusion, it is likely that there are additional genes regulated by C/EBPβ whose protein products promote this function independent of REDD1. And as noted, shCEBPB expression in PC3 cells only reduced REDD1 2-fold, likely allowing autophagosome formation, albeit at a slower rate. However, we were able to partly rescue the defect in autophagy in shCEBPB cells by ectopic expression of REDD1. In particular, LC3-II was not increased by shCEBPB in the presence of exogenous REDD1, suggesting increased flux through autophagy. The latter effect suggests that in the presence of shCEBPB, where autophagosome–lysosome fusion is suppressed, ectopic expression of REDD1 may have a positive feedback on the autophagy pathway or additional activities beyond its ability to induce LC3-II.

During periods of metabolic stress such as exercise, starvation, or hypoxia, REDD1 is critical for autophagosome maturation by inducing LC3-II and regulating proper autophagosome size [11]. *REDD1* transcript levels were not increased 16 hrs after 25 nM bortezomib treatment despite elevation of C/EBPβ LAP, indicating that bortezomib interferes with C/EBPβ induction of *REDD1* transcription, and protein levels were increased more than two-fold at this time point, suggesting stabilization of REDD1 protein. Consistent with the idea that bortezomib affects REDD1 protein levels independent of C/EBPβ, knockdown of *CEBPB* did not prevent up-regulation of REDD1 protein by bortezomib, though basal levels were reduced. We also found that LC3-II proteins were up-regulated within 4 hours of bortezomib treatment. However, in cells expressing shCEBPB, LC3-II levels showed only modest changes relative to control cells. These findings suggest that regulation of basal *REDD1* transcript and protein levels by C/EBPβ is crucial for LC3B processing early after proteasome inhibitor treatment.

Proteasome inhibition increases cytosolic and endoplasmic reticulum (ER) protein content and promotes amino acid deprivation [42,48]. These events activate the amino acid sensitive protein kinase GCN2 and the unfolded protein response in the ER, leading to phosphorylation of eIF2α and suppression of protein translation [49]. Autophagy is thought to promote cell survival in cells treated with proteasome inhibitors by restoring amino acid homeostasis and suppressing ER stress [42]. We found that PCa cells treated with bortezomib show a dynamic regulation of autophagy that was dependent upon C/EBPβ expression. Suppression of autolysosome catabolism has been reported in ovarian and breast cancer cell lines treated with bortezomib, owing to a decrease in cathepsins D and B [50,51]. The conclusions of these studies are consistent with our findings which showed strong up-regulation of *p62* and *ULK1* but suppression of *Cathepsin L* (not shown) in PC3 cells treated with bortezomib.

Finally, our findings also show decreased cell and tumor growth rates in PC3 cells lacking C/EBPβ coincident with a decline in basal autophagy. This decline in autophagy could explain the poor growth rates of cells lacking C/EBPβ as autophagy maintains cellular fitness by turnover of damaged organelles and long-lived proteins [44]. We also found that suppression of C/EBPβ increased PC3 cell and tumor susceptibility to bortezomib. Further, CQ did not additively increase PC3 cell death following bortezomib treatment, indicating that bortezomib-induced cell death was related to inhibition of autophagy. These results are in accordance with previous studies wherein combined autophagy and proteasome blockade additively increased tumor cell killing [52–54]. In particular, our results implicate C/EBPβ as a key mediator of autophagy in PCa and validate

the utility of targeting C/EBPβ in combination with bortezomib or other proteasome inhibitors as a novel approach to PCa therapy.

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Authors' contributions

DJB, SKK, IPP, and ADF conceived and designed the research. TB and JZ generated shCEBPB and shNTV constructs and cell lines. JM conducted the autophagosome–lysosome fusion analysis. DJB conducted the balance of the experiments. DJB and ADF wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

None to declare.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.03.005.

References

[1] M.J. Morris, W.K. Kelly, S. Slovin, C. Ryan, C. Eicher, G. Heller, et al., A phase II trial of bortezomib and prednisone for castration resistant metastatic prostate cancer, J. Urol. 178 (2007) 2378–2383.
[2] R. Dreicer, D. Petrylak, D. Agus, I. Webb, B. Roth, Phase I/II study of bortezomib plus docetaxel in patients with advanced androgen-independent prostate cancer, Clin. Cancer Res. 13 (2007) 1208–1215.
[3] J.D. Hainsworth, A.A. Meluch, D.R. Spigel, J. Barton Jr., L. Simons, C. Meng, et al., Weekly docetaxel and bortezomib as first-line treatment for patients with hormone-refractory prostate cancer: a Minnie Pearl Cancer Research Network phase II trial, Clin. Genitourin. Cancer 5 (2007) 278–283.
[4] S.G. Zhao, W.C. Jackson, V. Kothari, M.J. Schipper, N. Erho, J.R. Evans, et al., High-throughput transcriptomic analysis nominates proteasomal genes as age-specific biomarkers and therapeutic targets in prostate cancer, Prostate Cancer Prostatic Dis. 18 (2015) 229–236.
[5] L. Galluzzi, F. Pietrocola, J.M. Bravo-San Pedro, R.K. Amaravadi, E.H. Baehrecke, F. Cecconi, et al., Autophagy in malignant transformation and cancer progression, EMBO J. 34 (2015) 856–880.
[6] E.L. Axe, S.A. Walker, M. Manifava, P. Chandra, H.L. Roderick, A. Habermann, et al., Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum, J. Cell Biol. 182 (2008) 685–701.
[7] C.A. Lamb, T. Yoshimori, S.A. Tooze, The autophagosome: origins unknown, biogenesis complex, Nat. Rev. Mol. Cell Biol. 14 (2013) 759–774.
[8] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, et al., LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[9] H. Weidberg, E. Shvets, T. Shpilka, F. Shimron, V. Shinder, Z. Elazar, LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis, EMBO J. 29 (2010) 1792–1802.
[10] K. Satoo, N.N. Noda, H. Kumeta, Y. Fujioka, N. Mizushima, Y. Ohsumi, et al., The structure of Atg4B-LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy, EMBO J. 28 (2009) 1341–1350.
[11] S. Qiao, M. Dennis, X. Song, D.D. Vadysirisack, D. Salunke, Z. Nash, et al., A REDD1/TXNIP pro-oxidant complex regulates ATG4B activity to control stress-induced autophagy and sustain exercise capacity, Nat. Commun. 6 (2015) 7014.
[12] S. Pankiv, T.H. Clausen, T. Lamark, A. Brech, J.A. Bruun, H. Outzen, et al., p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (2007) 24131–24145.
[13] V. Kirkin, T. Lamark, Y.S. Sou, G. Bjørkøy, J.L. Nunn, J.A. Bruun, et al., A role for NBR1 in autophagosomal degradation of ubiquitinated substrates, Mol. Cell 33 (2009) 505–516.
[14] S. Geisler, K.M. Holmström, D. Skujat, F.C. Fiesel, O.C. Rothfuss, P.J. Kahle, et al., PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1, Nat. Cell Biol. 12 (2010) 119–131.
[15] H.G. Nguyen, J.C. Yang, H.J. Kung, X.B. Shi, D. Tilki, P.N. Lara Jr., et al., Targeting autophagy overcomes enzalutamide resistance in castration-resistant prostate cancer cells and improves therapeutic response in a xenograft model, Oncogene 33 (2014) 4521–4530.
[16] Q. Tan, A.M. Joshua, J.K. Saggar, M. Yu, M. Wang, N. Kanga, et al., Effect of pantoprazole to enhance activity of docetaxel against human tumour xenografts by inhibiting autophagy, Br. J. Cancer 112 (2015) 832–840.
[17] A. Modernelli, V. Naponelli, M. Giovanna Troglio, M. Bonacini, I. Ramazzina, S. Bettuzzi, et al., EGCG antagonizes Bortezomib cytotoxicity in prostate cancer cells by an autophagic mechanism, Sci. Rep. 5 (2015) 15270.
[18] M. Zhang, J. He, Z. Liu, Y. Lu, Y. Zheng, H. Li, et al., Anti-β_2-microglobulin monoclonal antibodies overcome bortezomib resistance in multiple myeloma by inhibiting autophagy, Oncotarget 6 (2015) 8567–8578.
[19] D.T. Vogl, E.A. Stadtmauer, K.S. Tan, D.F. Heitjan, L.E. Davis, L. Pontiggia, et al., Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma, Autophagy 10 (2014) 1380–1390.
[20] K. Zhu, K. Dunner Jr., D.J. McConkey, Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells, Oncogene 29 (2010) 451–462.
[21] W. B'chir, A.C. Maurin, V. Carraro, J. Averous, C. Jousse, Y. Muranishi, et al., The eIF2α/ATF4 pathway is essential for stress-induced autophagy gene expression, Nucleic Acids Res. 41 (2013) 7683–7699.
[22] C. Settembre, C. Di Malta, V.A. Polito, M. Garcia Arencibia, F. Vetrini, S. Erdin, et al., TFEB links autophagy to lysosomal biogenesis, Science 332 (2011) 1429–1433.
[23] D. Ma, S. Panda, J.D. Lin, Temporal orchestration of circadian autophagy rhythm by C/EBPβ, EMBO J. 30 (2011) 4642–4651.
[24] L. Guo, J.X. Huang, Y. Liu, X. Li, S.R. Zhou, S.W. Qian, et al., Transactivation of Atg4b by C/EBPβ promotes autophagy to facilitate adipogenesis, Mol. Cell. Biol. 33 (2013) 3180–3190.
[25] C.M. Shirley, J. Chen, M. Shamay, H. Li, C.A. Zahnow, S.D. Hayward, et al., Bortezomib induction of C/EBPβ mediates Epstein-Barr virus lytic activation in Burkitt lymphoma, Blood 117 (2011) 6297–6303.
[26] E.S. Hungness, B.W. Robb, G.J. Luo, T.A. Pritts, D.D. Hershko, P.O. Hasselgren, Proteasome inhibitors activate the transcription factors C/EBP-beta and delta in human intestinal epithelial cells, Biochem. Biophys. Res. Commun. 290 (2002) 469–474.
[27] J.J. Chen, W.C. Huang, C.C. Chen, Transcriptional regulation of cyclooxygenase-2 in response to proteasome inhibitors involves reactive oxygen species-mediated signaling pathway and recruitment of CCAAT/enhancer-binding protein delta and CREB-binding protein, Mol. Biol. Cell 16 (2005) 5579–5591.
[28] M.M. Tomayko, C.P. Reynolds, Determination of subcutaneous tumor size in athymic (nude) mice, Cancer Chemother. Pharmacol. 24 (1989) 148–154.
[29] D.J. Barakat, J. Zhang, T. Barberi, S.R. Denmeade, A.D. Friedman, I. Paz-Priel, CCAAT/enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells, Oncogene 34 (2015) 5912–5922.
[30] O. Topaloglu, P.J. Hurley, O. Yildirim, C.I. Civin, F. Bunz, Improved methods for the generation of human gene knockout and knockin cell lines, Nucleic Acids Res. 33 (2005) e158.
[31] I. Paz-Priel, S. Houng, J. Dooher, A.D. Friedman, C/EBPα and C/EBPα oncoproteins regulate nfkb1 and displace histone deacetylases from NF-κB p50 homodimers to induce NF-κB target genes, Blood 117 (2011) 4085–4094.
[32] S. Kimura, T. Noda, T. Yoshimori, Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3, Autophagy 3 (2007) 452–460.
[33] Y. Narain, A. Wyttenbach, J. Rankin, R.A. Furlong, D.C. Rubinsztein, A molecular investigation of true dominance in Huntington's disease, J. Med. Genet. 36 (1999) 739–746.
[34] D.J. Klionsky, F.C. Abdalla, H. Abeliovich, R.T. Abraham, A. Acevedo-Arozena, K. Adeli, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (2012) 445–544.
[35] L. Guo, X. Li, J.X. Huang, H.Y. Huang, Y.Y. Zhang, S.W. Qian, et al., Histone demethylase Kdm4b functions as a co-factor of C/EBPβ to promote mitotic clonal expansion during differentiation of 3T3-L1 preadipocytes, Cell Death Differ. 19 (2012) 1917–1927.
[36] K.A. Christensen, J.T. Myers, J.A. Swanson, pH-dependent regulation of lysosomal calcium in macrophages, J. Cell Sci. 115 (2002) 599–607.
[37] C. Mauvezin, P. Nagy, G. Juhász, T.P. Neufeld, Autophagosome-lysosome fusion is independent of V-ATPase-mediated acidification, Nat. Commun. 6 (2015) 7007.
[38] I.G. Ganley, H. du Lam, J. Wang, X. Ding, S. Chen, X. Jiang, ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy, J. Biol. Chem. 284 (2009) 12297–12305.
[39] L. Lin, T.M. Stringfield, X. Shi, Y. Chen, Arsenite induces a cell stress-response gene, RTP801, through reactive oxygen species and transcription factors Elk-1 and CCAAT/enhancer-binding protein, Biochem. J. 392 (2005) 93–102.
[40] H.O. Jin, S.K. Seo, S.H. Woo, E.S. Kim, H.C. Lee, D.H. Yoo, et al., Activating transcription factor 4 and CCAAT/enhancer-binding protein-beta negatively regulate the mammalian target of rapamycin via Redd1 expression in response to oxidative and endoplasmic reticulum stress, Free Radic. Biol. Med. 46 (2009) 1158–1167.

[41] C. Malagelada, E.J. Ryu, S.C. Biswas, V. Jackson-Lewis, L.A. Greene, RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation, J. Neurosci. 26 (2006) 9996–10005.
[42] A. Suraweera, C. Münch, A. Hanssum, A. Bertolotti, Failure of amino acid homeostasis causes cell death following proteasome inhibition, Mol. Cell 48 (2012) 242–253.
[43] D.R. Fels, J. Ye, A.T. Segan, S.J. Kridel, M. Spiotto, M. Olson, et al., Preferential cytotoxicity of bortezomib toward hypoxic tumor cells via overactivation of endoplasmic reticulum stress pathways, Cancer Res. 68 (2008) 9323–9330.
[44] F. Baixauli, C. López-Otín, M. Mittelbrunn, Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness, Front. Immunol. 5 (2014) 403.
[45] C.A. Surmacz, A.R. Pösö, G.E. Mortimore, Regulation of lysosomal fusion during deprivation-induced autophagy in perfused rat liver, Biochem. J. 242 (1987) 453–458.
[46] J. Kovacs, A.P. Karpati, Regression of autophagic vacuoles in mouse pancreatic cells: a morphometric study of the effect of methylamine and chloroquine followed by cycloheximide treatment, Cell Biol. Int. Rep. 13 (1989) 805–811.
[47] J. Pizarro-Cerdá, M. Desjardins, E. Moreno, S. Akira, J.P. Gorvel, Modulation of endocytosis in nuclear factor IL-6(-/-) macrophages is responsible for a high susceptibility to intracellular bacterial infection, J. Immunol. 162 (1999) 3519–3526.
[48] K.T. Bush, A.L. Goldberg, S.K. Nigam, Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance, J. Biol. Chem. 272 (1997) 9086–9092.
[49] D. Senft, Z.A. Ronai, UPR, autophagy, and mitochondria crosstalk underlies the ER stress response, Trends Biochem. Sci. 40 (2015) 141–148.
[50] C. Kao, A. Chao, C.L. Tsai, W.C. Chuang, W.P. Huang, G.C. Chen, et al., Bortezomib enhances cancer cell death by blocking the autophagic flux through stimulating ERK phosphorylation, Cell Death Dis. 5 (2014) e1510.
[51] S. Periyasamy-Thandavan, W.H. Jackson, J.S. Samaddar, B. Erickson, J.R. Barrett, L. Raney, et al., Bortezomib blocks the catabolic process of autophagy via a cathepsin-dependent mechanism, affects endoplasmic reticulum stress and induces caspase-dependent cell death in antiestrogen-sensitive and resistant ER+ breast cancer cells, Autophagy 6 (2010) 19–35.
[52] S. Chen, Y. Zhang, L. Zhou, Y. Leng, H. Lin, M. Kmieciak, et al., A Bim-targeting strategy overcomes adaptive bortezomib resistance in myeloma through a novel link between autophagy and apoptosis, Blood 124 (2014) 2687–2697.
[53] W.X. Ding, H.M. Ni, W. Gao, X. Chen, J.H. Kang, D.B. Stolz, et al., Oncogenic transformation confers a selective susceptibility to the combined suppression of the proteasome and autophagy, Mol. Cancer Ther. 8 (2009) 2036–2045.
[54] B. Hui, Y.H. Shi, Z.B. Ding, J. Zhou, C.Y. Gu, Y.F. Peng, et al., Proteasome inhibitor interacts synergistically with autophagy inhibitor to suppress proliferation and induce apoptosis in hepatocellular carcinoma, Cancer 118 (2012) 5560–5571.

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ORIGINAL ARTICLE

CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells

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The processes associated with transition to castration-resistant prostate cancer (PC) growth are not well understood. Cellular senescence is a stable cell cycle arrest that occurs in response to sublethal stress. It is often overcome in malignant transformation to confer a survival advantage. CCAAT/Enhancer Binding Protein (C/EBP) β function is frequently deregulated in human malignancies and interestingly, androgen-sensitive PC cells express primarily the liver-enriched inhibitory protein isoform. We found that C/EBPβ expression is negatively regulated by androgen receptor (AR) activity and that treatment of androgen-sensitive cell lines with anti-androgens increases C/EBPβ mRNA and protein levels. Accordingly, we also find that C/EBPβ levels are significantly elevated in primary PC samples from castration-resistant compared with therapy-naive patients. Chromatin immunoprecipitation demonstrated enhanced binding of the AR to the proximal promoter of the *CEBPB* gene in the presence of dihydroxytestosterone. Upon androgen deprivation, induction of C/EBPβ is facilitated by active transcription as evident by increased histone 3 acetylation at the C/EBPβ promoter. Also, the androgen agonist R1881 suppresses the activity of a *CEBPB* promoter reporter. Loss of C/EBPβ expression prevents growth arrest following androgen deprivation or anti-androgen challenge. Accordingly, suppression of C/EBPβ under low androgen conditions results in reduced expression of senescence-associated secretory genes, significantly decreased number of cells displaying heterochromatin foci and increased numbers of Ki67-positive cells. Ectopic expression of C/EBPβ caused pronounced morphological changes, reduced PC cell growth and increased the number of senescent LNCaP cells. Lastly, we found that senescence contributes to PC cell survival under androgen deprivation, and C/EBPβ-deficient cells were significantly more susceptible to killing by cytotoxic chemotherapy following androgen deprivation. Our data demonstrate that upregulation of C/EBPβ is critical for complete maintenance of androgen deprivation-induced senescence and that targeting C/EBPβ expression may synergize with anti-androgen or chemotherapy in eradicating PC.

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INTRODUCTION

Prostate cancer (PC) is the most prevalent malignancy in adult men in the United States.[1] Although early detection and treatment of localized disease is often curative, PC remains a leading cause of cancer death. Anti-androgen therapy is the most effective approach in patients with advanced disease and induces significant responses in almost all patients.[2] However, androgen deprivation achieved by pharmacologic or surgical castration results in only limited apoptosis of tumor cells[3,4] and accordingly only partial tumor regression. Indeed, after a period of disease control, most patients develop castration-resistant growth and PC progression, which is responsible for the majority of the morbidity and mortality associated with this disease.[2] Identifying mechanisms that engender castration resistance is crucial for the design of future therapeutic strategies. Progress has been made understanding the mechanisms associated with eventual emergence of castration-resistant PC (CRPC). However, less is known about the early adaptation associated with androgen deprivation.[5–7]

Members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors are characterized by a conserved C-terminus, which contains both a DNA-binding basic region and leucine-zipper, collectively referred to as the bZIP domain. C/EBPβ is a widely expressed transcription factor that promotes proliferation or terminal differentiation and growth arrest in several different cell types.[8] These opposing functions seem to be regulated by the expression of different C/EBPβ translational isoforms from three in-frame start codons within an intron-less mRNA.[9,10] The two high molecular weight C/EBPβ isoforms, termed liver-enriched activating proteins (LAP and LAP*), contain N-terminal transactivation domains, whereas the liver-enriched inhibitory protein lacks these transactivation domains. Liver-enriched inhibitory protein can dominantly inhibit LAPs and other C/EBP members via heterodimerization or by recruiting transcriptional repressors.[11] C/EBPβ activity affects several facets of PC disease progression. C/EBPβ regulates the expression of steroidogenic genes including StAR and cytochrome p450 aromatase,[12,13] and its activity is modulated in response to dihydrotestosterone, estrogen and progesterone.[14–17] It has also been suggested that C/EBPβ can act as a co-repressor of the androgen receptor (AR) in PC.[18,19] Although C/EBPβ is not detected in healthy prostate, luminal epithelial cells upregulate C/EBPβ in the case of proliferative-inflammatory atrophy, a precursor to PC, [20] and C/EBPβ participates in the regulation of metastatic genes and PC cell survival.[21,22] However, the contribution of C/EBPβ to the emergence of castration-resistant growth has not been previously investigated.

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Cellular senescence is a stable cell cycle arrest that occurs in response to a variety of intrinsic and extrinsic sublethal stress stimuli.[23,24] Accumulating data point to an important role of senescence in cancer progression.[23,24] Recently, several groups demonstrated that in response to androgen deprivation, PC cells undergo senescence, and that the acquisition of senescence is associated with emergence of castrate-resistant growth.[5–7] In other lineages, C/EBPβ and its downstream target genes are critical for the induction and maintenance of oncogene-induced senescence, associated with overexpression of activated Ras or BRAF.[8,25,26] C/EBPβ can directly bind to target gene promoters and enhancers to induce senescence-associated factors IL-6 and IL-8, but can also suppress E2F-1 target genes and induce growth arrest dependent on E2F:pRb.[8]

We now demonstrate that upon androgen deprivation, C/EBPβ is rapidly upregulated in androgen-sensitive PC cells and that AR binds to and suppresses the C/EBPβ proximal promoter. Increased expression of C/EBPβ under these conditions is necessary for acquisition of the senescent phenotype. Accordingly, preventing C/EBPβ upregulation increases the susceptibility of PC cells to apoptosis induced by chemotherapy.

RESULTS

CRPC is associated with increased *CEBPB*

To determine whether C/EBPβ levels correlate with human PC progression, we interrogated the Oncomine database.[27] In the Grasso *et al*.[28] data set that included gene expression patterns from 28 benign prostate tissues, 59 localized PC and 35 CRPC samples, *CEBPB* expression was significantly ($P < 1.9 \times 10^{-6}$) elevated in CRPC compared with localized disease (Figures 1a and b).

Inhibition of AR induces *CEBPB* transcription

Treatment of LNCaP cells with the synthetic AR agonist R1881 for 24 h results in a dose-dependent 2.5-fold decrease in *CEBPB* mRNA and protein expression (Figures 2a and b), and as expected, prostate-specific antigen transcript levels increased under these conditions. Conversely, culturing LNCaP cells in androgen-depleted media (ADM) for 7 days resulted in a significant 3.8-fold increase in C/EBPβ expression (Figure 2c). Pharmacologic inhibition of the AR using bicalutamide resulted in a dose-dependent rise in *CEBPB* transcript abundance, achieving a 7.5-fold increase at the highest dose tested (Figure 2d). Accordingly, we detected increased protein levels of C/EBPβ in both LNCaP and LAPC4 cells treated with bicalutamide or flutamide (Figure 2e). As bicalutamide or flutamide may have an AR agonist effect, we also tested the effect of enzalutamide, which does not have agonistic effects. Similar to bicalutamide, incubation with 20 μM enzalutamide resulted in increased C/EBPβ levels (Figure 2f). *CEBPB* RNA levels were rapidly upregulated within 4 h of exposure of LNCaP cells to bicalutamide (Figure 2g).

To assess binding of AR to the *CEBPB* promoter, LNCaP cells were cultured in full media and subjected to chromatin immunoprecipitation analysis. Precipitated DNA was amplified using primers spanning the proximal (−131 to − 242 bp) or distal (−2098 to − 1983 bp) regions of the human *CEBPB* promoter. We observed AR binding to the proximal but not the distal region (Figure 3a). Next, CEBPB-luc, containing proximal promoter region (−888 to +64) linked to a luciferase reporter, was co-transfected into LNCaP or DU145 PC cells with CMV-β-galactosidase as internal control. Reproducibly, luciferase activity significantly decreased by 2.5-fold in LNCaP cells cultured with 1 nM R1881 for 24 h compared with vehicle control (Figure 3b). This effect on *CEBPB* promoter activation was mediated by the AR as R1881 did not reduce luciferase activity in similarly transfected DU145 cells which lack AR. Treatment of LNCaP cells with bicalutamide for 4 h induced acetylation of histone H3 bound to the *CEBPB* proximal promoter, whereas culture with dihydrotestosterone suppressed this mark of active transcription (Figure 3c). Importantly, we did not observe significant changes in the half-life of *CEBPB* RNA in response to bicalutamide, indicating that the stability of *CEBPB* transcripts was unaffected (Figure 3d). Collectively, these results show that the AR suppresses transcription of *CEBPB*.

Ectopic expression of C/EBPβ suppresses LNCaP cell growth

Because C/EBPβ expression was inversely regulated by AR activity, an essential signal for PC cell growth, we next evaluated whether ectopic expression induces growth arrest similar to androgen deprivation. C/EBPβ was inducibly expressed in LNCaP cells using the Tet-regulated transposon-based piggyBac vector[29] and cells with stable integration of the transgene or an empty vector (PB-TRE) control were selected by puromycin (2 mg/ml). Treatment of LNCaP PB-CEBPB cells with doxycycline for 5 days induced an increase in the expression of C/EBPβ 2.5-fold (Figure 4a), similar to the increase seen in LNCaP cells cultured in enzalutamide. C/EBPβ forced expression was associated with increased levels of the cell cycle inhibitors $p16^{INK4A}$ and $p15^{INK4B}$ and a flattened morphology (Figures 4a and b). Next, we tested the effect of C/EBPβ overexpression on LNCaP cell proliferation. Equal numbers of LNCaP PB-CEBPB or PB-TRE control cells were seeded in doxycycline-containing media and enumerated after 3 and 5 days. Ectopic C/EBPβ expression resulted in a significant 2.3-fold decreased rate of proliferation (Figure 4c) and a significantly increased number of Ki67-negative cells 5 days after doxycycline treatment (Figure 4d) without increased cell death (Figure 4e). These data demonstrate that elevated levels of C/EBPβ are sufficient to suppress the growth of androgen-sensitive PC cells.

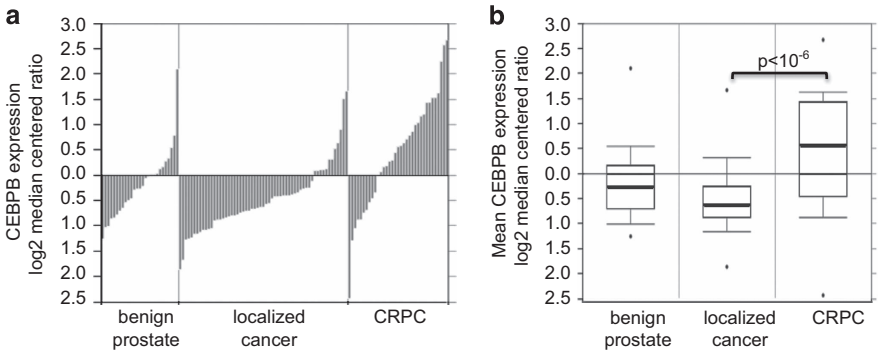

Figure 1. C/EBPβ expression increases in CRPC. Individual patient (**a**) and mean (**b**) *CEBPB* expression as log2 median centered ratio for benign prostate, localized PC and CRPC.

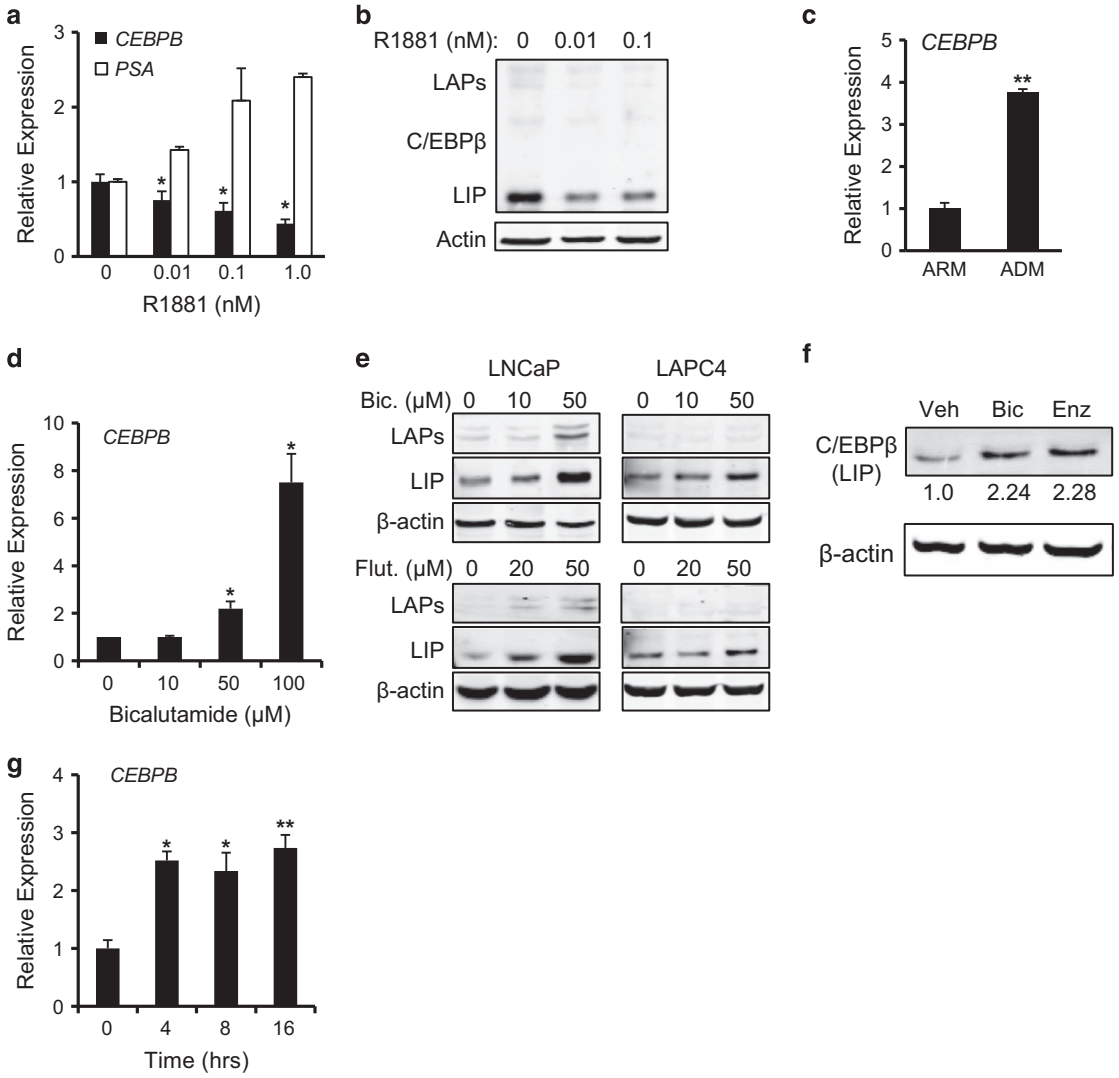

Figure 2. C/EBPβ expression is regulated by AR activity in PC cell lines. LNCaP cells were cultured in the indicated concentrations of R1881 for 24 h and RNA (**a**) or protein (**b**) were analyzed for the expression of the indicated gene products. (**c**) LNCaP cells were cultured in ARM or ADM for 9 days and *CEBPB* RNA levels analyzed. Mean and s.d. from three independent experiments are shown. (**d**) *CEBPB* transcripts levels in LNCaP cells cultured with the indicated concentration of bicalutamide were measured in three independent experiments using qRT–PCR. (**e**) LNCaP or LAPC4 cells were cultured with the indicated doses of bicalutamide (Bic), flutamide (Flut) or (**f**) enzalutamide (enz) for 24 h, and the cell lysates were subjected to western blotting. Representative gels with relative band intensity values are shown. (**g**) LNCaP cells were exposed to bicalutamide at 50 μM and C/EBPβ expression was assessed at the indicated time points. The average normalized *CEBPB* transcripts levels from three independent experiments are shown. $^{*}P < 0.01$, $^{**}P < 0.001$.

C/EBPβ is required for complete maintenance of androgen-deprivation-induced growth arrest

Persistence of PC cells under androgen-deprived conditions is an initial step towards development of castration-resistant growth. Upon androgen deprivation, LNCaP cells undergo cell cycle arrest and enter a senescent state.[5–7] Importantly, androgen deprivation-induced senescence is only partly reversible and cells continue to display senescence markers and poor proliferation after re-exposure to androgen.[6] Because C/EBPβ upregulation was associated with androgen deprivation and its expression sufficient to suppress LNCaP proliferation, we next targeted C/EBPβ to test its function in androgen-deprivation-induced growth arrest. We utilized two independent methods: inducible shRNA and transcription activator-like effector nucleases (TALENs).[30,31] We designed a pair of TALENs to target the *CEBPB* gene, transfected LNCaP cells, and screened individual clones for C/EBPβ expression. Subclone 6, was identified as having *CEBPB* knockdown (presumably due to incomplete targeting of all alleles in the polyploid LNCaP cells) (Figure 5a), and used in our subsequent experiments. A complete stable deletion of all *CEBPB* alleles could not be achieved, suggesting C/EBPβ plays a critical role for cell survival. As control we employed subclone 1 in which C/EBPβ expression is similar to that observed in parental cells. In addition, C/EBPβ expression was effectively knocked down in LNCaP cells utilizing a doxycycline-inducible shRNA against *CEBPB* (shCEBPB) compared with a non-targeting vector control (shNTV) (Figure 5b). We used flow cytometry to investigate the effect of C/EBPβ depletion on PC cell cycle distribution (Figure 5c). For these studies, we employed cells expressing the inducible shRNA as stable deletion of C/EBPβ via TALEN required adaptation to low C/EBPβ levels. Cells expressing shCEBPB or shNTV both went into G1 cell cycle arrest when cultured in ADM (Figure 5c).

The growth arrest induced by androgen deprivation is associated with changes in cell cycle inhibitors including $p16^{INK4A}$, $p15^{INK4B}$ and $p21^{WAF1}$. Because C/EBPβ has been shown to regulate the expression of these genes, we next evaluated their

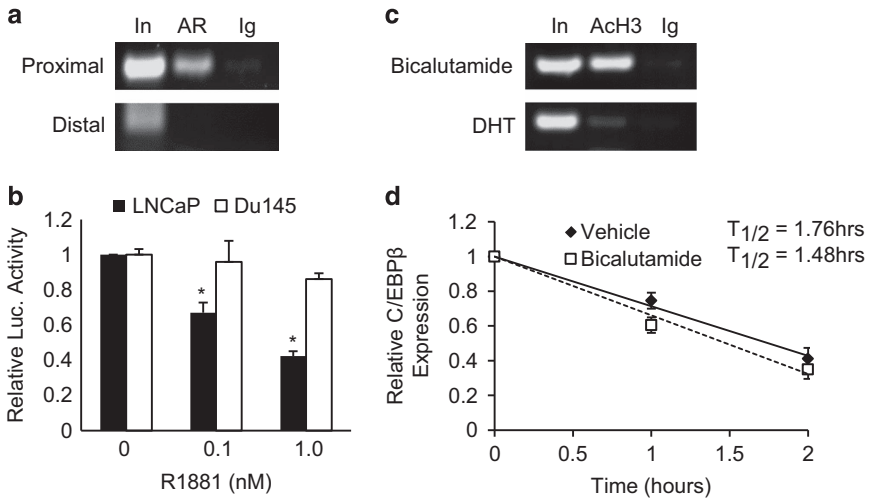

Figure 3. AR binds and represses the CEBPB gene. (**a**) LNCaP cells were subjected to chromatin immunoprecipitation analysis with antibodies against AR or normal rabbit IgG (Ig). PCR was used to amplify DNA fragments centered at − 187 bp (proximal) or an upstream fragment centered at − 2040 bp (distal) of the *CEBPB* gene. (**b**) LNCaP or DU145 cells were transiently transfected with CEBPB-Luc and cultured with vehicle or R1881 at the indicated dose. Fold activation of the reporter relative to vehicle-treated cells was determined after normalization to β-galactosidase activity. The average from three independent experiments is presented. (**c**) LNCaP cells were cultured in the presence of bicalutamide (50 μM) or dihydroxytestosterone (DHT) (10 nM) for 4 h and subjected to chromatin immunoprecipitation using antibodies against acetylated histone 3 (AcH3) or normal rabbit IgG (Ig). A representative gel of PCR-amplified proximal promoter DNA fragment is shown. (**d**) LNCaP cells were cultured in the presence of vehicle or bicalutamide (50 μM) and after 4 h actinomycin D (5 μg/ml) was added to the media. Total cellular RNA was harvested at the indicated time points, reverse-transcribed to cDNA and relative *CEBPB* transcript levels were measured using qRT–PCR. The slope of a linear best-fit line for each group was determined to calculate the *CEBPB* RNA half-life, based on three repetitions.

expression in LNCaP cells following androgen deprivation. The levels of $p16^{INK4A}$, $p15^{INK4B}$ and $p21^{WAF1}$ proteins were diminished in C/EBPβ-deficient cells compared with shNTV cultured in androgen-replete media (ARM) or ADM (Figure 5d). In contrast to $p15^{INK4B}$ and $p21^{WAF1}$, $p16^{INK4A}$ protein levels mildly increased in shCEBPB cells following culture in ADM, compared with culture in ARM. However, $p16^{INK4A}$ levels in shCEBPB cells are markedly lower compared with shNTV control cells cultured in either ARM or ADM (Figure 5d).

To evaluate whether C/EBPβ is required to maintain growth arrest of LNCaP cells challenged by androgen deprivation, we pre-cultured LNCaP cells harboring shNTV or shC/EBPβ in ADM or ARM with doxycycline for 7 days, re-seeded equal number of cells in ARM and enumerated viable cells 5 days later (Figure 6a). As expected from the observed inhibition of G1 to S cell cycle progression (Figure 5c), culture in ADM for 7 days resulted in diminished cell accumulation (not shown). Although in ARM, the rate of proliferation of shCEBPB cells was modestly lower than shNTV cells, C/EBPβ knockdown was associated with a better, although incomplete, recovery of proliferation after pre-culture in ADM (Figure 6b).

Charcoal-stripped fetal bovine serum (FBS) lacks androgen and multiple other growth factors. To define the specific effect of androgen depletion, LNCaP cells harboring shNTV or shCEBPB were pre-cultured with doxycycline and bicalutamide, enzalutimide or vehicle for 4 days, and subsequently re-plated at equal numbers in ARM without inhibitors (Figure 6a). In contrast to shNTV cells, which demonstrated twofold decreased recovery, *CEBPB*-depleted cells completely recovered their proliferation once released from AR inhibition (Figure 6c). To better reflect recovery and adjust for the different proliferation rate in ARM of shCEBP compared with shNTV cells, we also plotted these data as a ratio of growth of cells pre-cultured in ADM, bicalutamide or enzalutimide to cells pre-cultured in ARM (Figure 6d). This normalization highlights the recovery of proliferation of shCEBPB compared with shNTV cells in each experiment, indicating that C/EBPβ is required for maintenance of complete growth arrest induced by androgen deprivation and its suppression alleviates the phenotype, at least in part.

C/EBPβ elevation induces senescence

Because C/EBPβ seemed to play a role in maintaining growth arrest following androgen deprivation, we next evaluated whether overexpression of C/EBPβ was sufficient to induce senescence in LNCaP cells. Senescent cells are characterized by an increase in cell volume, granularity and lysosomal mass indicated by senescence-associated β-galactosidase (SA-β-gal) activity.[32] Compared with control PB-TRE, PB-CEBPB cells had a significant increase in the number SA-β-gal-positive cells and the level of cell granularity, as assessed by side scatter (Figures 7a and b). Expression of several secreted gene products is elevated in senescent cells and is referred to as the senescence-associated secretory phenotype.[24] Release of these secreted factors promotes paracrine growth arrest, and C/EBPβ was shown to be central to induction of senescence-associated secretory phenotype genes.[25,33,34] Accordingly, overexpression of C/EBPβ led to a significant increase in the transcript levels of two senescence-associated secretory phenotype-associated genes, *IL8* and *IGFBP3* (Figure 7c). Similarly, we observed increased IGFBP3 and IL8 levels upon androgen deprivation, which was abrogated by either *CEBPB* shRNA or TALEN targeting (Figures 7d and e). Another feature of senescent cells is accumulation of tightly packed heterochromatin foci characterized by increased di- or trimethylated histone 3 on lysine 9 (H3K9me2, H3K9me3).[35] When cultured in ADM, LNCaP-shNTV cells display a twofold increase in the number of heterochromatin foci-positive cells. In contrast, we did not observe an increase in heterochromatin foci in shCEBPB cells cultured under similar conditions (Figure 7f). Senescent cells exit the cell cycle, and stain negative for Ki67. A similar proportion of shNTV or shCEBPB cells expressed Ki67 in ARM. However, the proportion of Ki67-negative cells after 1 week of culture in ADM was twofold lower in cells lacking C/EBPβ (Figure 7g). Together, these data indicate that in PC cells C/EBPβ is necessary for induction of senescence upon androgen deprivation.

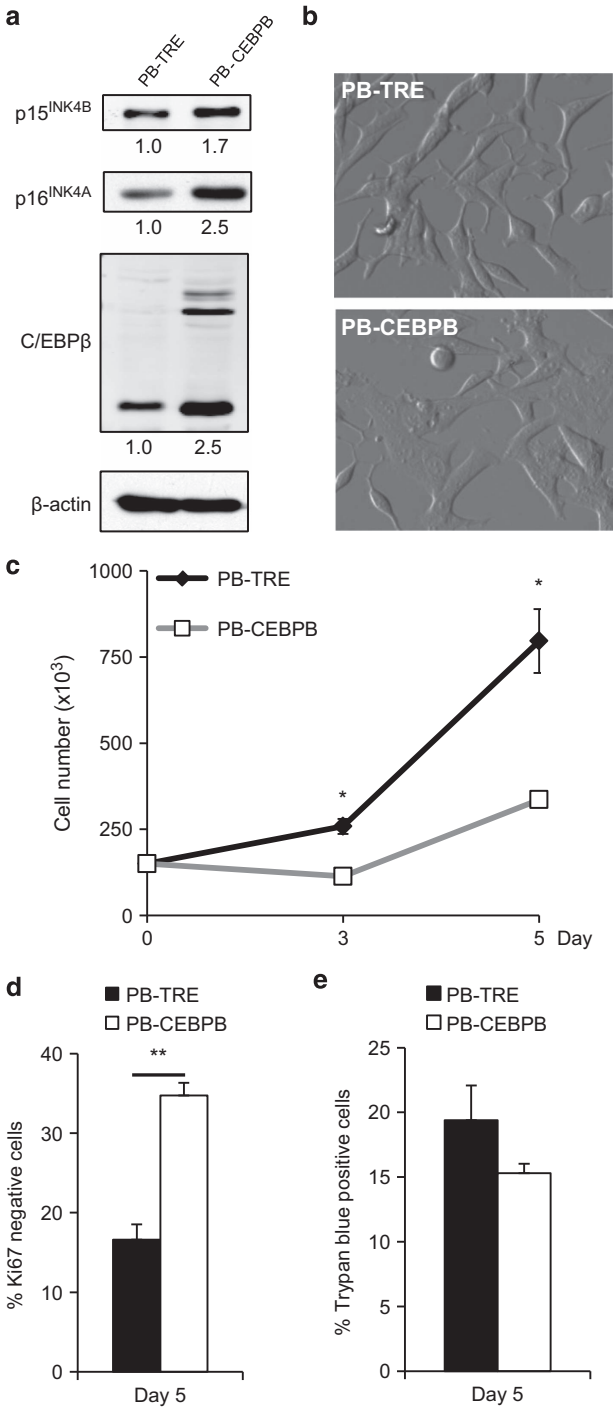

Figure 4. Ectopic expression of C/EBPβ suppresses LNCaP cell growth. (**a**) LNCaP PB-CEBPB and PB-TRE control cells were cultured for 5 days in 0.5 μg/ml doxycycline. Cell lysates were obtained and a representative western blot analysis for C/EBPβ, $p15^{INK4B}$ and $p16^{INK4A}$ is shown. Numbers below the blots indicate the relative band density. (**b**) Phase-contrast images of LNCaP PB-TRE and PB-CEBPB cells after 5 days in culture with 0.5 μg/ml doxycycline. (**c**) 1.5E5 LNCaP PB-CEBPB or PB-TRE cells were seeded in media containing 0.5 μg/ml doxycycline. Cells were enumerated on day 3 and 5. (**d**) Cells were cultured under similar conditions and the Ki67 expression was analyzed after 5 days of doxycycline treatment. (**e**) The number of dead cells on day 5 was assessed by trypan blue exclusion. All graphs represent the average of three experiments, error bars: s.e.m.; $*P < 0.01$; $**P < 0.02$.

Cellular senescence engenders a pro-survival phenotype. Given rapid induction of C/EBPβ in the absence of AR signaling and its role in directing senescence, we evaluated whether targeting C/EBPβ synergizes with anti-androgen agents or chemotherapy in killing PC cells. Culture of LNCaP shNTV and shCEBPB in ADM (Figure 8a) or exposing them to bicalutamide (Figure 8b) increased the number of dead cells relative to ARM cultures. However, there was no difference in cell viability between shNTV and shCEBPB cells (Figures 8a and b). However, after pre-culture in ADM, treatment with docetaxel or etoposide induced a significant 68% or 55% increase in cell death, respectively, in LNCaP cells harboring shCEBPB compared with shNTV (Figure 8c). Together, these data demonstrate that C/EBPβ promotes a pro-survival, drug-resistant phenotype during androgen deprivation.

DISCUSSION

Accumulating evidence points to a strong connection between senescence and tumor progression.[23,24,36] Oncogene-induced senescence promotes the eventual emergence of subpopulations of aggressive, malignant cells and thus may be considered a tumor-promoting state. In PC, androgen-deprivation-induced senescence promotes the development of tumor progression and resistance to apoptosis,[5] fostering the emergence of cancer-initiating cells.[37] Increased numbers of senescent cells have been observed in tissue sections from tumors in patients that had been treated with neoadjuvant androgen deprivation therapy.[7] Also, cellular senescence induced by androgen deprivation dramatically increases reactive oxygen species and DNA double-strand breaks, and leads to the outgrowth of hormone-refractory populations in cultured LNCaP cells.[5] CRPC emerges as a result of multiple adaptations, including AR gene amplification, abnormal AR activation or enhanced steroidogenesis.[2] These changes are acquired and propagated as a result of selective pressure exerted on PC cells by an androgen-poor milieu while cells are protected by the androgen-deprivation-induced senescence state. Previous reports have suggested that androgen deprivation leads to a senescent state in both LNCaP and LAPC4 cells, but the mechanism by which these cells become senescent was not well described.[5–7] Here, we demonstrate that PC cells respond to androgen withdrawal by upregulating *CEBPB* transcription, that loss of C/EBPβ lead to a reduction in the number of senescent cells following androgen deprivation, and that ectopic expression of C/EBPβ induces the expression of senescent markers indicating that C/EBPβ plays a central role in cellular senescence induced by androgen deprivation, and that impeding the senescent response via inhibition of C/EBPβ expression keeps PC cells susceptible to chemotherapy, validating C/EBPβ as a therapeutic target in androgen-dependent PC.

Probing the Oncomine database revealed elevated expression of *CEBPB* in human CRPC samples. As CRPC is often characterized by active AR signaling, this finding may seemingly be at odds with our *in vitro* data showing that AR activity suppresses C/EBPβ expression. Several potential explanations may account for elevated C/EBPβ levels despite active AR signaling in CRPC. It has been demonstrated that there is substantial divergence in AR gene targets when comparing castrate-resistant to androgen-sensitive cells.[38] Further, androgen deprivation or castration resistance is associated with decreased AR occupancy on repressive DNA elements,[39] and the expression of many AR-repressed genes increases in castrate-resistant cells.[39] Finally, although castrate-resistant cells often exhibit increased levels of AR, AR signaling relative to androgen-sensitive PC cells may not increase because of diminished ligand levels. Therefore, C/EBPβ de-repression may persist in cells that have developed castration resistance.

Our data demonstrate that inhibition of AR leads to rapid upregulation of *CEBPB* RNA, loss of AR interaction with the *CEBPB*

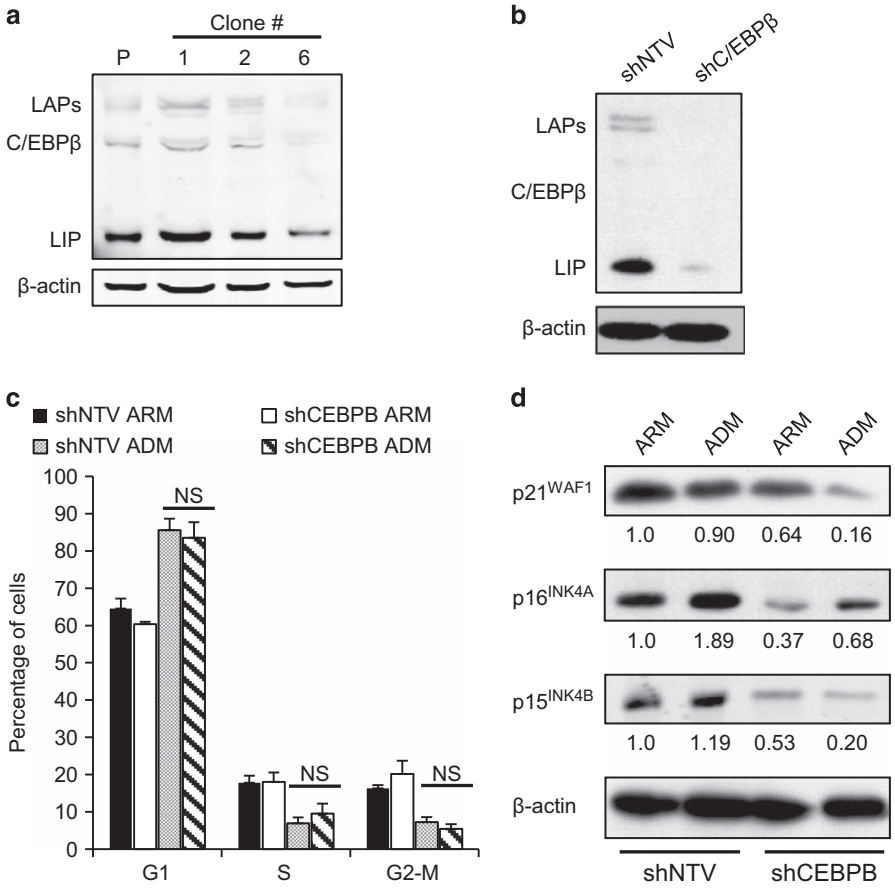

Figure 5. C/EBPβ is required for high expression levels of cell cycle inhibitors upon AR inhibition. (**a**) Total cellular proteins extracted from parental (P) LNCaP cells or individual clones transfected with TALEN-C/EBPβ were subjected to western blotting using C/EBPβ or actin antibodies. (**b**) LNCaP cells harboring an inducible shRNA against C/EBPβ (shC/EBPβ) or non-targeting vector (NTV) were cultured with doxycycline (200 ng/ml) for 48 h and protein extracts were subjected to immunoblotting with the indicated antibodies. (**c**) LNCaP cells harboring shCEBPB or non-targeting vector (shNTV) cultured with doxycycline (200 ng/ml) in ARM or ADM for 7 days, were stained with propidium iodide and DNA content was analyzed by flow cytometry. Mean distribution of cells in G1, S or G2/M from three experiments. (**d**) LNCaP cells expressing shNTV or shCEBPB were cultured in ARM or ADM for 7 days, and the expression of the indicated cell cycle regulators was assessed by western blotting.

promoter and increased promoter H3K acetylation. To our knowledge, this is the first demonstration that *CEBPB* is a direct AR transcriptional repressive target. This observation is consistent with previous reports showing that AR can inhibit gene expression through interaction with transcriptional co-repressors at proximal promoter regions.[40–42] Derepression of *CEBPB* occurs within 4 h of exposure to anti-androgens. Conversely, treatment with AR agonist R1881 results in diminished expression of C/EBPβ and suppression of the activity of a *CEBPB* promoter luciferase reporter. Accordingly, exposure to dihydrotestosterone leads to a decrease in activating AcH3 histone marks on the promoter. These findings indicate that AR suppresses *CEBPB* expression directly through regulation of the promoter. Examination of the *CEBPB* promoter sequence did not identify an androgen response element, suggesting indirect binding of AR. In other contexts, AR directly interacts with Sp1 to regulate gene expression in the absence of an androgen response element making Sp1 a potential mediator of AR regulation of *CEBPB*.[43,44]

Treatment of LNCaP cells with anti-androgens or culture in hormone-depleted media leads to G1 arrest and cellular senescence. PC cells require AR signaling for transition from G1 to S, and accordingly, we did not observe continued proliferation of cells that had been cultured in hormone-depleted media or with anti-androgens regardless of *CEBPB* knockdown. Androgen-deprivation-induced senescence had a profound long-lived effect on PC cell proliferation in the presence of normal C/EBPβ levels even after reintroduction of androgens, as previously observed.[6] We found that after androgen deprivation, C/EBPβ deficiency allowed LNCaP cells to resume proliferation when re-seeded in ARM. Thus, C/EBPβ plays an important role in the complete maintenance of senescent growth arrest induced by androgen deprivation.

Senescent cells develop unique secretory paracrine activities, conferring a pro-malignant microenvironment by secreting an array of cytokines and proinflammatory mediators.[24,45,46] Our results raise the possibility that C/EBPβ also promotes the expression of senescence-associated secretory genes such as IL-8 and IGFBP3 and the cell cycle inhibitors $p21^{WAF1}$ and $p15^{INK4B}$. IL-8 signaling has been shown to promote cell survival, angiogenesis and senescence in pre-clinical models of PC.[8,47,48] It activates the PI3K-AKT-mTOR pathway, which is critical for cell survival during androgen deprivation.[49] IGFBP3 is strongly upregulated following androgen deprivation and promotes tumor growth in a mouse PC model.[50,51] In PC patients, the percentage of cells positive for $p15^{INK4B}$ was shown to increase with tumor grade.[52–54] Expression of $p21^{WAF1}$ correlates with a worsened prognosis both before and after androgen deprivation therapy, and *in vitro* studies of $p21^{WAF1}$ have shown that it suppresses

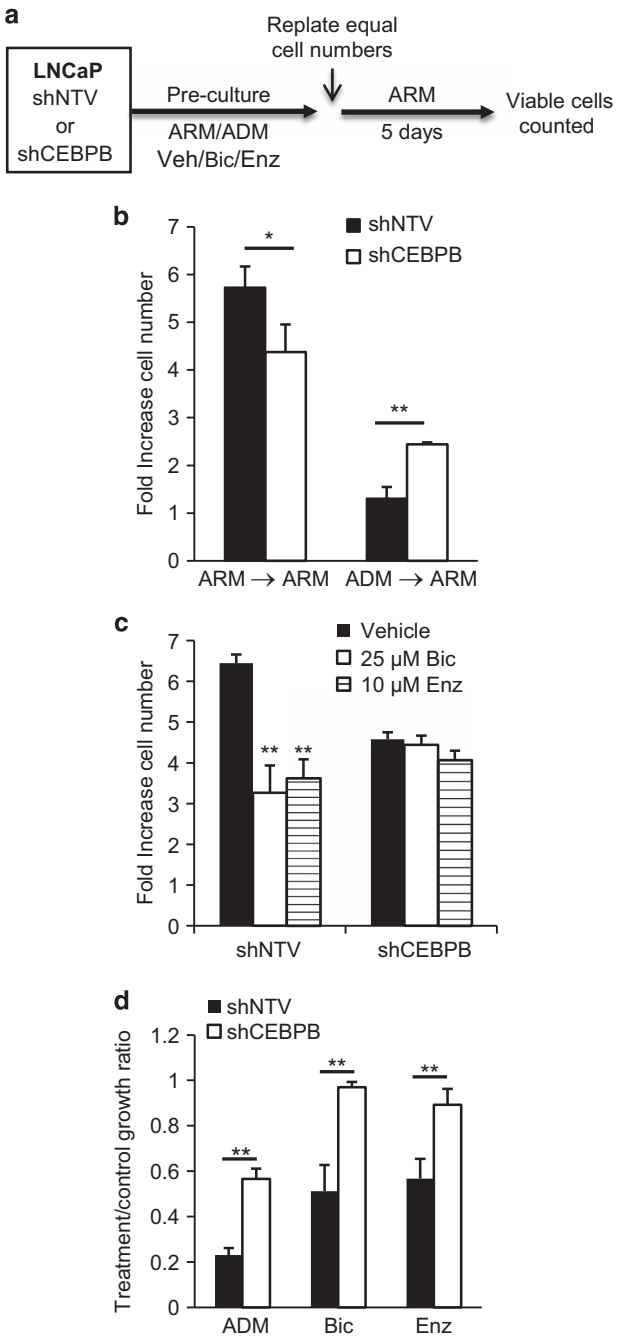

Figure 6. C/EBPβ is required for complete maintenance of growth arrest induced by AR inhibition in LNCaP cells. (**a**) LNCaP cells expressing shCEBPB or shNTV were pre-cultured in doxycycline under the indicated conditions or in ARM control media, 1.5E5 cells from each pre-culture condition were replated and enumerated after 5 days, as depicted in the diagram. (**b**) After 7 days pre-culture in ARM or ADM, equal number of shCEBPB or shNTV LNCaP cells were re-plated in ARM in a 6-well dish and counted 5 days later. The average fold increase in cell number is shown. (**c**) Similarly, cells were pre-cultured for 4 days with bicalutamide (Bic), enzalutamide (Enz) or vehicle control and doxycycline, re-plated in ARM and enumerated after 5 days as described above. (**d**) The relative growth ratio of cells pre-cultured in ADM, bicalutamide (Bic) or enzalutamide (Enz) over cells cultured in ARM control conditions. Bar graphs represent the mean of at least three experiments ± s.e.m.; $^{*}P < 0.05$; $^{**}P < 0.005$.

apoptotic response to chemotherapy.[55–57] These findings are consistent with our results showing that C/EBPβ-deficient cells, with decreased $p21^{WAF1}$ and $p15^{INK4B}$ levels, were more sensitive to chemotherapy post androgen withdrawal. Increased $p16^{INK4A}$ expression is not essential to androgen-induced senescence,[5] and we see suppression of $p16^{INK4A}$ in C/EBPβ-deficient LNCaP cells. Overall, C/EBPβ promotes PC senescence and thereby potentially chemo-resistance and progression to castration-resistant growth through multiple transcriptional targets during androgen deprivation therapy (Figure 8d).

Our delineation of *CEBPB* upregulation in human hormone-refractory PC further support the concept that C/EBPβ-dependent induction of senescence during androgen blockade promotes castration-resistant progression by providing the opportunity to respond to the selective pressure of anti-androgen therapy. Importantly, these data indicate the potential utility of targeting C/EBPβ in combination with androgen deprivation for novel PC therapy.

MATERIALS AND METHODS

Cell lines and plasmids

LNCaP cells were maintained in RPMI media without phenol red with 10% heat-inactivated FBS (HI-FBS) (Hyclone Laboratories, Logan, UT, USA) supplemented with penicilin/streptomycin. LAPC4 cells were maintained in Iscove's modified Dulbecco's media (IMDM) supplemented with 15% HI-FBS, 1 nM dihydrotestosterone and penicillin/streptomycin. DU145 cells were maintained in RPMI with 10% HI-FBS, and 293T cells were cultured in Dulbecco modified Eagle medium with 10% HI-FBS. Cells were grown in a humidified incubator maintained at 37 °C with 5% CO_2. Cells were split 1:4 and were used until passage 40. Cells transduced with pTRIPZ-shRNA or transfected with pPB-TRE-Puro were maintained in tetracycline-screened FBS (Hyclone Laboratories). For androgen deprivation, cells were cultured in phenol red-free media supplemented with 10% charcoal-stripped FBS (Hyclone Laboratories). AR was blocked using enazlutamide (Selleckchem, Houston, TX, USA) or bicalutamide (Sigma-Aldrich, St Louis, MO, USA).

pTRIPZ-shRNA (Open Biosystems, Lafayette, CO, USA) lentiviral vectors were generated as described[58] and stably transduced cells were selected with puromycin (2 μg/ml) after 48 h. Expression of shRNA was induced by treating cells with 200 ng/ml doxycycline, replaced every 48 h and confirmed by fluorescence microscopy detection of RFP.

The pPB-TRE-Puro plasmid (kindly provided by Jolene Ooi and Pentao Liu) contains a multiple cloning site downstream of a TRE element, and a CAG promoter upstream of rtTA, IRES and apuromycin resistance gene. The mouse *Cebpb* ORF including the 3′UTR (1–1400) was ligated as *BamH*I/*Not*I fragment into the *Bgl*II/*Not*I-digested pPB-TRE-Puro plasmid[29] to generate the pPB-CEBPB which was confirmed by sequencing. Stable PB-TRE and PB-CEBPB cell lines were generated by transfecting equal parts pCMV-hyperpiggybase and piggybac vectors by lipofection.

Western blotting

Protein samples from whole-cell lysates and nuclear extracts were prepared and subjected to western blotting as previously described.[59] After blocking with Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE, USA), membranes were incubated overnight with the following primary antibodies: $p15^{INK4B}$ (M-20), C/EBPβ (C-19), AR (N-20) (Santa Cruz Biotechnology, Dallas, TX, USA), β-actin (AC15) (Sigma-Aldrich). For target protein detection, membranes were incubated with secondary antibodies, goat anti-mouse Alexafluor 670 (Life Technologies, Carlsbad, CA, USA) or goat anti-rabbit antiserum (LI-COR) and imaged on a Li-Cor Odyssey Fc infrared imaging system.

Quantitative real-time PCR

Total RNA was isolated from cells and first strand cDNA was synthesized as previously described.[58] β-actin transcript was used as a reference to normalize samples and relative expression was calculated as described.[58] Each sample was assayed in triplicates and each experiment was repeated at least three times. Oligonucleotides were custom ordered from Sigma-Aldrich, and their sequences are presented in Table 1.

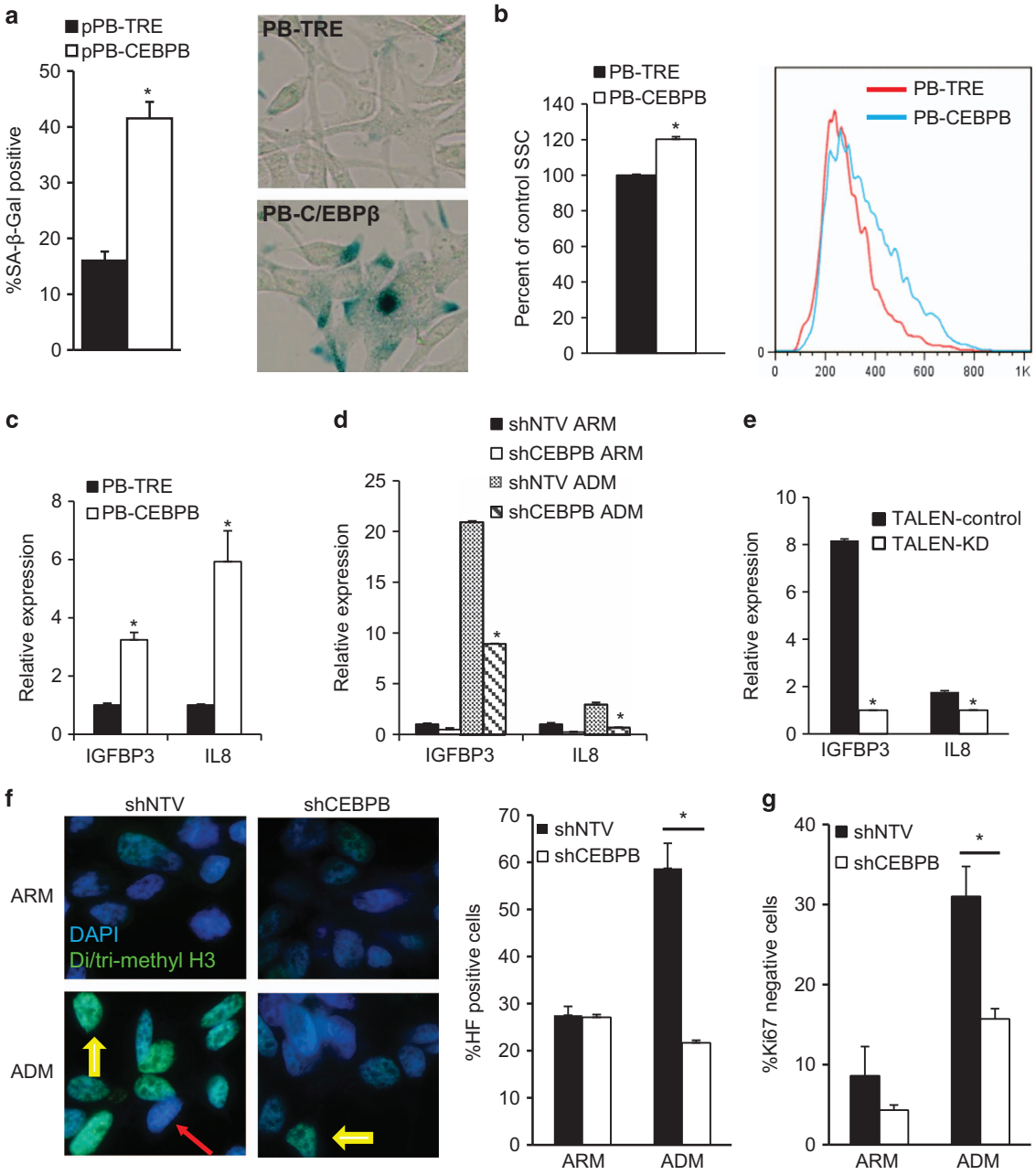

Figure 7. C/EBPβ is required for androgen-deprivation-induced senescence. (**a**) The percent of SA-β-gal-positive PB-TRE or PB-CEBPB cells was quantified after 5 days of culture in 0.5 μg/ml doxycycline. An average from three experiments (left) and representative images are shown (right). (**b**) Quantification of cell granularity by the median side scatter value of G1 PB-TRE and PB-CEBPB cells 5 days after seeding in doxycycline. A representative histogram is shown on the right. (**c**) IL8 and IGFBP3 expression were quantified by qRT–PCR in PB-TRE and PB-CEBPB cells cultured under the above conditions. Conversely, total cellular RNA was extracted from shNTV or shCEBPB (**d**), or TALEN-control or TALEN-KD (**e**) LNCaP cells after 7 days in culture in ARM or ADM and transcript levels of IL8 and IGFBP3 were assessed by qRT–PCR. (**f**) Expression of an shRNA against *CEBPB* or non-targeting vector (shNTV) control were induced by doxycycline in LNCaP cells cultured in ARM or ADM. After 7 days, cells were stained for senescence-associated heterochromatin foci (HF) (yellow arrows). Red arrow points to HF-negative cells. Representative photomicrographs are shown (left, ×600 magnification). The average and s.e. of three independent counts is shown (right panel). *$P < 0.001$. (**g**) Ki67 was quantified using flow cytometry in shNTV or shCEBPB LNCaP cells cultured in ARM or ADM for 7 days and the average proportion of Ki67 negative cells from three experiments is presented. *$P < 0.01$.

Luciferase reporter assays

Using PCR, a DNA fragment from −888 to +64 base pairs (bp) relative to the initiation of transcription (Ensembl database) of the human *CEBPB* promoter was cloned as a *Bam*HI/*Mlu*I fragment into *Bgl*II/*Mlu*I-digested pGL3-luciferase reporter vector (Promega, Madison, WI, USA) to generate CEBPB-Luc that was confirmed by Sanger sequencing. LNCaP or DU145 cells were seeded in 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Each well was co-transfected with 1.4 μg of the reporter plasmid and 10 ng of CMV-β-galactosidase as an internal control. Twenty four hours after transfection, cells were treated with vehicle (0.01% ethanol) or the androgen agonist R1881 (Sigma-Aldrich) and after additional 24 h, cells were lysed (Reporter Lysis Buffer, Promega) and assayed for luciferase and β-galactosidase activity. Fold activation relative to the vehicle-treated cells after correction for β-galactosidase activity was determined. Lysates from non-transfected cells were used as baseline.

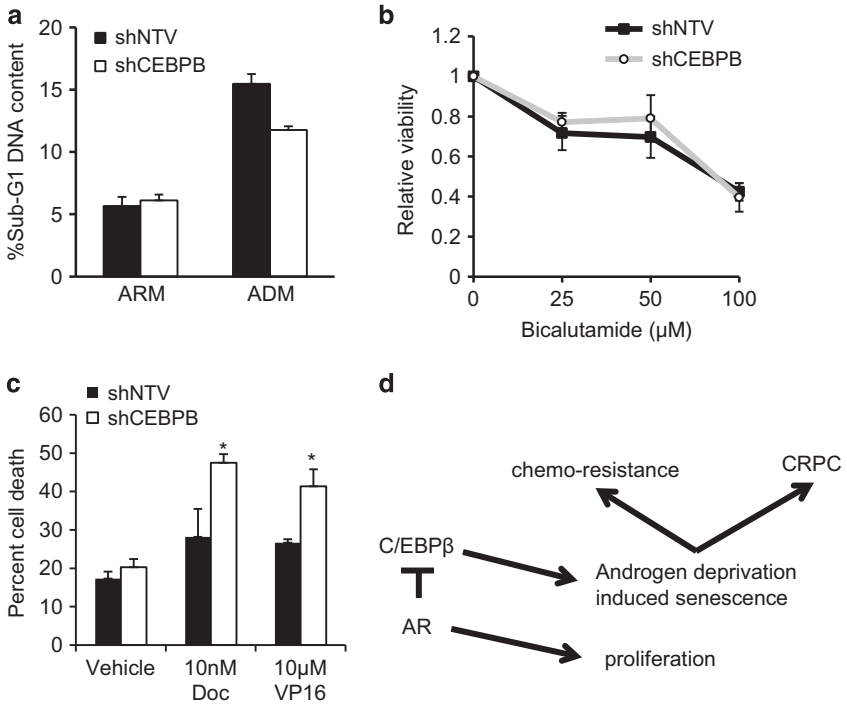

Figure 8. C/EBPβ protects from chemotherapy following androgen deprivation. (**a**) LNCaP cells were cultured for 1 week in ARM or ADM, and cell death was evaluated flow cytometric analysis of sub-G1 DNA content. (**b**) LNCaP shNTV or shCEBPB lines cultured in ADM were exposed to bicalutamide at the indicated dose for 48 h and relative cell viability was evaluated by the WST-1 assay. The average cell viability from three independent experiments is shown. (**c**) LNCaP lines harboring shNTV or shCEBPB were cultured for 7 days in ADM, re-plated in ARM, and treated with vehicle control, 10 nM docetaxel (Doc), or 10 μM etoposide (VP16) for 48 h. Cell survival was assessed by trypan blue exclusion and the average percentage of dead cells from three independent experiments is shown. (**d**) Model of the regulation of C/EBPβ expression by AR. Upon AR inhibition, C/EBPβ expression will increase to promote senescence, chemoresistence and emergence of CRPC.

Table 1. Primers used for ChIP and RNA analysis

Gene	*Forward sequence*	*Reverse sequence*
ChIP		
CEBPB proximal	GGCCGCCCTTATAAATAACC	TATTAGTGAGGGGGCTGGTG
CEBPB upstream	ATAATGGTGGCTGGCGATAG	CCTTCCTCACTGCAAAATGG
Real-time PCR		
TMPRSS2	AATCCCCATCCGGGACAGT	AGGAGTCGCACTCTATCCCA
PSA	GCAGCATTGAACCAGAGGAG	AGAACTGGGGAGGCTTGAGT
ACTB	GACCTGGCTGGCCGGGACCT	GGCCATCTCTTGCTCGAAGT
GAPDH	CCACCCATGGCAAATTCC	GATGGGATTTCCATTGATGACA
CEBPB	AAACTCTCTGCTTCTCCCTCTGC	CTGACAGTTACACGTGGGTTGC
IL8	TCTGGCAACCCTAGTCTGCT	GCTTCCACATGTCCTCACAA
BDNF	GCGTGTGTGACAGTATTAGT	CTGGGTAGTTCGGCACTGGG
CGA	GCGGTGGAAGAGCCATCAT	TCTGTGGCTTCACCACTTTTCTC
IGFBP3	GCCAGCGCTACAAAGTTGAC	ATGTGTACACCCCTGGGACT
P16 INK4A	CCGAATAGTTACGGTCGGAGG	CACCAGCGTGTCCAGGAAG
P15 INK4B	GAGGCGCGCGATCCAG	CACCAGCGTGTCCAGGAAG
P21 CIP1/WAF1	ACTCTCAGGGTCGAAAACGG	GATGTAGAGCGGGCCTTTGA

Abbreviation: ChIP, chromatin immunoprecipitation.

TALEN construction and CEBPB gene editing

TALEN DNA constructs targeting the human *CEBPB* ORF were constructed using the Golden Gate Talen assembly kit (Addgene, Cambridge, MA, USA).[30] Targeting sequences were designed using the Cornell University TAL Effector Nucleotide Targeter 2.0 web-based software. Golden Gate assembly of the repeat-variable di-residue sequence was performed according to the manufacturer's instructions, and the completed TALEN pairs were ligated into the pTAL3 vector. The complete TALEN ORF including the repeat-variable and FokI domains was excised using *Xho*I and *Apa*I restriction endonuceases and ligated into the pcDNA3.1(+) vector. LNCaP cells cotransfected with TALEN expression vectors targeting *CEBPB* were seeded in 96-well dishes, and individual clones were screened for C/EBPβ expression by western blotting.

DNA content analysis and flow cytometry

Cell cycle analysis by DNA content was performed as previously described.[60] Ki67-expressing cells were identified by flow cytometry using APC-anti-Ki67 antibody (BioLegend, San Diego, CA, USA). Flow cytometry analysis was performed using a BD FACSCalibur machine (BD Biosciences, San Jose, CA, USA). Sub-cellular debris and dead cells were gated out and singlet discrimination was performed by gating on FL2-A and FL2-W channels and data were interpreted using FloJo Cytometric Analytical software (TreeStar, Ashland, OR, USA).

Chromatin immunoprecipitation

5E6 LNCaP cells were used in each chromatin immunoprecipitation reaction as previously described,[58] using antibodies against C/EBPβ, AR, rabbit IgG (Santa Cruz Biotechnology) or acetylated histone H3 (06–599) (Millipore, Billerica, MA, USA). DNA fragments corresponding to the promoters of interest were detected by PCR using the primers presented in Table 1.

Cell viability and proliferation assays

Viability was determined using the WST-1 assay (Roche, Indianapolis, IN, USA). Briefly, cells were seeded into 96-well plates, allowed to adhere for 48 h and then treated for an additional 48 h. WST-1 reagent (Roche) was directly added to the wells, and after incubation, absorbance was read at 450 nm using a Bio-Rad (Hercules, CA, USA) Microplate Reader Model 680. The 670 nm reference absorbance and readings from blank wells containing only cell culture media and DMSO (vehicle) were subtracted from experimental wells. Relative viability was determined by dividing absorbance readings from vehicle-treated wells. For cell proliferation, LNCaP cells were grown for 7 days in androgen depleted media. Cells were trypsinized, stained with Trypan blue dye and viable cells were enumerated using a hemocytometer. 1.5E5 cells per well were seeded into 6-well plates in ARM. After 5 days, cells were similarly enumerated.

SA-β-gal chromogenic assay

SA-β-gal-positive cells were stained using the chromogenic assay as described.[61] Five random fields of view were imaged on a Leica E600 (Leica, Buffalo Grove, IL, USA) microscope by brightfield microscopy at ×200 magnification. Positive cells were identified as those containing blue precipitate throughout the cytoplasm.

Immunofluorescent staining and heterochromatin foci quantification

LNCaP cells were seeded onto poly-D-lysine-coated glass coverslips and following treatment, were fixed in 4% paraformaldehyde. Cells were washed and incubated in permeabilization buffer (TBS, 2% BSA, 0.5% Triton-X 100, 0.1% sodium azide). After blocking, cells were incubated with anti-di/trimethyl H3K9 (1:250, Cell Signaling Technology, Danvers, MA, USA), washed and incubated with goat anti-mouse-Alexafluor 488-conjugated secondary antibody (Life Technologies). Cells were washed and mounted on glass slides for analysis by fluorescence microscopy. Heterochromatin foci were imaged on a Leica E800 fluorescence microscope with a CCD camera and imaged at ×630 magnification with an oil immersion objective. Fluorescent micrographs of heterochromatin foci from six to seven random fields of view were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA). Intensely stained nuclei with multiple fluorescent foci that colocalized with DAPI staining were counted as positive cells containing heterochromatin foci.

Statistical analysis

Statistical comparison of two groups of samples was conducted using the Student's *t*-test. Comparisons of multiple groups of samples was performed using the analysis of variance followed by multiple comparisons with the Student's *t*-test and the Holm-Bonferonni Correction ($\alpha/(n-k+1)$, where n = number of comparisons and k = rank of *P*-value).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DJB designed and performed the research, analyzed data and wrote the manuscript; JZ and TB performed the research; SRD designed the research and analyzed data; ADF designed the research, analyzed data and wrote the manuscript; IP-P designed and performed the research, analyzed data and wrote the manuscript.

REFERENCES

1 American Cancer Society. Cancer Facts and Figures 2013, 2013. http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-036845.pdf.
2 Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. *N Engl J Med* 2003; **349**: 366–381.
3 Denmeade SR, Isaacs JT. Activation of programmed (apoptotic) cell death for the treatment of prostate cancer. *Adv Pharmacol* 1996; **35**: 281–306.
4 Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* 1996; **28**: 251–265.
5 Burton DG, Giribaldi MG, Munoz A, Halvorsen K, Patel A, Jorda M *et al.* Androgen deprivation-induced senescence promotes outgrowth of androgen-refractory prostate cancer cells. *PLoS One* 2013; **8**: e68003.
6 Ewald JA, Desotelle JA, Church DR, Yang B, Huang W, Laurila TA *et al.* Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo. *Prostate* 2013; **73**: 337–345.
7 Pernicova Z, Slabakova E, Kharaishvili G, Bouchal J, Kral M, Kunicka Z *et al.* Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2. *Neoplasia* 2011; **13**: 526–536.
8 Sebastian T, Malik R, Thomas S, Sage J, Johnson PF. C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. *EMBO J* 2005; **24**: 3301–3312.
9 Calkhoven CF, Muller C, Leutz A. Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev* 2000; **14**: 1920–1932.
10 Timchenko NA, Welm AL, Lu X, Timchenko LT. CUG repeat binding protein (CUGBP1) interacts with the 5' region of C/EBPbeta mRNA and regulates translation of C/EBPbeta isoforms. *Nucleic Acids Res* 1999; **27**: 4517–4525.
11 Tanabe A, Kumahara C, Osada S, Nishihara T, Imagawa M. Gene expression of CCAAT/enhancer-binding protein delta mediated by autoregulation is repressed by related gene family proteins. *Biol Pharm Bull* 2000; **23**: 1424–1429.
12 Christenson LK, Johnson PF, McAllister JM, Strauss JF 3rd. CCAAT/enhancer-binding proteins regulate expression of the human steroidogenic acute regulatory protein (StAR) gene. *J Biol Chem* 1999; **274**: 26591–26598.
13 Zhou J, Gurates B, Yang S, Sebastian S, Bulun SE. Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. *Cancer Res* 2001; **61**: 2328–2334.
14 Bagchi MK, Mantena SR, Kannan A, Bagchi IC. Control of uterine cell proliferation and differentiation by C/EBPbeta: functional implications for establishment of early pregnancy. *Cell cycle* 2006; **5**: 922–925.
15 Boruk M, Savory JG, Hache RJ. AF-2-dependent potentiation of CCAAT enhancer binding protein beta-mediated transcriptional activation by glucocorticoid receptor. *Mol Endocrinol* 1998; **12**: 1749–1763.
16 Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC *et al.* C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. *Proc Natl Acad Sci U S A* 2006; **103**: 1870–1875.
17 Stein B, Yang MX. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol* 1995; **15**: 4971–4979.
18 Zhang J, Gonit M, Salazar MD, Shatnawi A, Shemshedini L, Trumbly R *et al.* C/EBPalpha redirects androgen receptor signaling through a unique bimodal interaction. *Oncogene* 2010; **29**: 723–738.
19 Jia L, Berman BP, Jariwala U, Yan X, Cogan JP, Walters A *et al.* Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. *PLoS One* 2008; **3**: e3645.
20 Wang W, Bergh A, Damber JE. Increased expression of CCAAT/enhancer-binding protein beta in proliferative inflammatory atrophy of the prostate: relation with the expression of COX-2, the androgen receptor, and presence of focal chronic inflammation. *Prostate* 2007; **67**: 1238–1246.
21 Kim MH, Fields J. Translationally regulated C/EBP beta isoform expression upregulates metastatic genes in hormone-independent prostate cancer cells. *Prostate* 2008; **68**: 1362–1371.
22 Kim MH, Minton AZ, Agrawal V. C/EBPbeta regulates metastatic gene expression and confers TNF-alpha resistance to prostate cancer cells. *Prostate* 2009; **69**: 1435–1447.
23 Collado M, Serrano M. Senescence in tumours: evidence from mice and humans. *Nat Rev Cancer* 2010; **10**: 51–57.
24 Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010; **5**: 99–118.
25 Huggins CJ, Malik R, Lee S, Salotti J, Thomas S, Martin N *et al.* C/EBPgamma suppresses senescence and inflammatory gene expression by heterodimerizing with C/EBPbeta. *Mol Cell Biol* 2013; **33**: 3242–3258.
26 Orjalo AV, Bhaumik D, Gengler BK, Scott GK, Campisi J. Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. *Proc Natl Acad Sci U S A* 2009; **106**: 17031–17036.
27 Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB *et al.* Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia* 2007; **9**: 166–180.
28 Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP *et al.* The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 2012; **487**: 239–243.
29 Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A* 2011; **108**: 1531–1536.
30 Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C *et al.* Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 2011; **39**: e82.
31 Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG 2nd *et al.* In vivo genome editing using a high-efficiency TALEN system. *Nature* 2012; **491**: 114–118.
32 Kurz DJ, Decary S, Hong Y, Erusalimsky JD. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 2000; **113**: 3613–3622.
33 Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S *et al.* Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* 2008; **133**: 1006–1018.

34 Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ *et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* 2008; **133**: 1019–1031.
35 Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA *et al.* Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 2003; **113**: 703–716.
36 Lehmann BD, Paine MS, Brooks AM, McCubrey JA, Renegar RH, Wang R *et al.* Senescence-associated exosome release from human prostate cancer cells. *Cancer Res* 2008; **68**: 7864–7871.
37 Cahu J, Bustany S, Sola B. Senescence-associated secretory phenotype favors the emergence of cancer stem-like cells. *Cell Death Dis* 2012; **3**: e446.
38 Sharma NL, Massie CE, Ramos-Montoya A, Zecchini V, Scott HE, Lamb AD *et al.* The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man. *Cancer Cell* 2013; **23**: 35–47.
39 Cai C, He HH, Chen S, Coleman I, Wang H, Fang Z *et al.* Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. *Cancer Cell* 2011; **20**: 457–471.
40 Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002; **9**: 601–610.
41 Svensson C, Ceder J, Iglesias-Gato D, Chuan YC, Pang ST, Bjartell A *et al.* REST mediates androgen receptor actions on gene repression and predicts early recurrence of prostate cancer. *Nucleic Acids Res* 2013; **42**: 999–1015.
42 Svensson C, Ceder J, Iglesias-Gato D, Chuan YC, Pang ST, Bjartell A *et al.* REST mediates androgen receptor actions on gene repression and predicts early recurrence of prostate cancer. *Nucleic Acids Res* 2014; **42**: 999–1015.
43 Lu S, Jenster G, Epner DE. Androgen induction of cyclin-dependent kinase inhibitor p21 gene: role of androgen receptor and transcription factor Sp1 complex. *Mol Endocrinol* 2000; **14**: 753–760.
44 Yuan H, Gong A, Young CY. Involvement of transcription factor Sp1 in quercetin-mediated inhibitory effect on the androgen receptor in human prostate cancer cells. *Carcinogenesis* 2005; **26**: 793–801.
45 Bavik C, Coleman I, Dean JP, Knudsen B, Plymate S, Nelson PS. The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. *Cancer Res* 2006; **66**: 794–802.
46 Dean JP, Nelson PS. Profiling influences of senescent and aged fibroblasts on prostate carcinogenesis. *Br J Cancer* 2008; **98**: 245–249.
47 Manna S, Singha B, Phyo SA, Gatla HR, Chang TP, Sanacora S *et al.* Proteasome inhibition by bortezomib increases IL-8 expression in androgen-independent prostate cancer cells: the role of IKKalpha. *J Immunol* 2013; **191**: 2837–2846.
48 Seaton A, Scullin P, Maxwell PJ, Wilson C, Pettigrew J, Gallagher R *et al.* Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells via induction of androgen receptor expression and activation. *Carcinogenesis* 2008; **29**: 1148–1156.
49 MacManus CF, Pettigrew J, Seaton A, Wilson C, Maxwell PJ, Berlingeri S *et al.* Interleukin-8 signaling promotes translational regulation of cyclin D in androgen-independent prostate cancer cells. *Mol Cancer Res* 2007; **5**: 737–748.
50 Kawabata R, Oie S, Takahashi M, Kanayama H, Oka T, Itoh K. Up-regulation of insulin-like growth factor-binding protein 3 by 5-fluorouracil (5-FU) leads to the potent anti-proliferative effect of androgen deprivation therapy combined with 5-FU in human prostate cancer cell lines. *Int J Oncol* 2011; **38**: 1489–1500.
51 Mehta HH, Gao Q, Galet C, Paharkova V, Wan J, Said J *et al.* IGFBP-3 is a metastasis suppression gene in prostate cancer. *Cancer Res* 2011; **71**: 5154–5163.
52 Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC *et al.* Overexpression of the cell cycle inhibitor p16INK4A in high-grade prostatic intraepithelial neoplasia predicts early relapse in prostate cancer patients. *Clin Cancer Res* 2001; **7**: 544–550.
53 Lee CT, Capodieci P, Osman I, Fazzari M, Ferrara J, Scher HI *et al.* Overexpression of the cyclin-dependent kinase inhibitor p16 is associated with tumor recurrence in human prostate cancer. *Clin Cancer Res* 1999; **5**: 977–983.
54 Zhang Z, Rosen DG, Yao JL, Huang J, Liu J. Expression of p14ARF, p15INK4b, p16INK4a, and DCR2 increases during prostate cancer progression. *Mod Pathol* 2006; **19**: 1339–1343.
55 Baretton GB, Klenk U, Diebold J, Schmeller N, Lohrs U. Proliferation- and apoptosis-associated factors in advanced prostatic carcinomas before and after androgen deprivation therapy: prognostic significance of p21/WAF1/CIP1 expression. *Br J Cancer* 1999; **80**: 546–555.
56 Martinez LA, Yang J, Vazquez ES, Rodriguez-Vargas Mdel C, Olive M, Hsieh JT *et al.* p21 modulates threshold of apoptosis induced by DNA-damage and growth factor withdrawal in prostate cancer cells. *Carcinogenesis* 2002; **23**: 1289–1296.
57 Steinman RA, Johnson DE. p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. *Mol Med* 2000; **6**: 736–749.
58 Paz-Priel I, Houng S, Dooher J, Friedman AD. C/EBPalpha and C/EBPalpha oncoproteins regulate nfkb1 and displace histone deacetylases from NF-kappaB p50 homodimers to induce NF-kappaB target genes. *Blood* 2011; **117**: 4085–4094.
59 Paz-Priel I, Cai DH, Wang D, Kowalski J, Blackford A, Liu H *et al.* CCAAT/enhancer binding protein alpha (C/EBPalpha) and C/EBPalpha myeloid oncoproteins induce bcl-2 via interaction of their basic regions with nuclear factor-kappaB p50. *Mol Cancer Res* 2005; **3**: 585–596.
60 Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. *Blood* 1999; **94**: 560–571.
61 Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc* 2009; **4**: 1798–1806.